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**THE AETIOLOGY OF AMOEBIC GILL DISEASE (AGD) AND ASPECTS  
OF THE HOST IMMUNE RESPONSE TO INFECTION**

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### **Statement of originality**

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The work presented in this thesis is, to the best of my knowledge and belief, original and my own work, except as acknowledged in the text. I hereby declare that I have not submitted this material, either in whole or in part, for a degree at this or any other university.

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## Abstract

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Amoebic gill disease (AGD) is an ectoparasitic condition of some marine fish.

Atlantic salmon are highly susceptible to AGD and the cost of mitigation is a significant financial burden for the aquaculture industry, particularly in Tasmania, Australia. Despite a considerable research effort over the past 20 years, two fundamental questions remain unanswered:

1. Which species of amoeba causes AGD? Two species of amoebae, *Neoparamoeba pemaquidensis* and *Neoparamoeba branchiphila* have been isolated from the gills of AGD-affected fish. Based on morphology alone, either species may be associated with AGD yet all attempts to experimentally induce AGD with cultured strains of either species have been unsuccessful.
2. Why are Atlantic salmon highly susceptible to AGD and is this susceptibility linked to aberrant innate and adaptive immune responses?

In this thesis, the aetiology of AGD was resolved by identifying and characterising a new species of amoeba, *Neoparamoeba perurans* and demonstrating that this species is the only known aetiological agent of AGD globally. In so doing, the phylogeny of *Neoparamoeba* was resolved and the strict co-evolution of *Neoparamoeba* species with their endosymbiont, *Perkinsela amoebae*-like organism was confirmed as a defining characteristic amongst all members of the *Neoparamoeba*.

Following this, the mechanisms that underpin the susceptibility of Atlantic salmon to AGD were assessed. Global gene expression profiling the gills of AGD-affected fish revealed that transcripts associated with the immune response were almost universally down-regulated in AGD-lesions specifically. In AGD-affected tissue,

significant, coordinated down-regulation of the major histocompatibility complex class I (MHC I), and possibly the MHC II pathway-related genes occurred during the later stages of infection and appeared to be mediated by down-regulation of interferon-regulatory factor (IRF)-1, independent of interferon- $\alpha$ , interferon- $\gamma$  (IFN- $\gamma$ ) and IRF-2 expression. Stimulating AGD-lesions *ex vivo* with recombinant IFN- $\gamma$  failed to restore the expression of IRF-1 and the MHC I receptor molecule, thus confirming earlier observations that the MHC I antigen presentation pathway appears to be modulated independently of IFN- $\gamma$  in AGD lesions. Within the AGD lesion microenvironment, suppression of the MHC I and possibly the MHC II pathways may inhibit the development of acquired immunity and could explain the unusually high susceptibility of Atlantic salmon to AGD. Whilst the data are preliminary, the immunologically unresponsiveness of the AGD lesion microenvironment is possibly linked with a disruption in the NF- $\kappa$ B signalling pathway which may permit *N. perurans* to evade the host immune response. Finally it is proposed that an understanding of the mechanisms of localised immunosuppression will be particularly important for the development of new treatments for AGD since systemic immunostimulation may be ineffective without simultaneous disruption of the immune privilege-like microenvironment within AGD lesions.

## Statement of contribution by others

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The following people and institutions contributed to the publication of the work undertaken as part of this thesis:

I certify that the published manuscripts from this thesis are the product of my own work. I was responsible for performing all the experiments, collecting all the data in the lab using techniques described in the thesis, analysing the data, producing the draft manuscripts, managing all changes in response to the co-author's recommendations and managing the manuscripts through the review and publishing process. In accomplishing my research goals I did receive assistance and I hereby acknowledge the contribution of other researchers to my published manuscripts.

### *Paper 1 (Chapter 2)*

Young N. D. (60%), Crosbie P. B. B. (5%), Adams M. B. (5%), Nowak B. F. (10%) and Morrison R. N. (20%) (2007) *Neoparamoeba perurans* n. sp., an agent of amoebic gill disease of Atlantic salmon (*Salmo salar* L.). *International Journal for Parasitology* **37**, 1469-1481.

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***We the undersigned agree with the above stated “proportion of work undertaken” for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:***

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## List of abbreviations

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$\beta$ 2m	$\beta$ -2-microglobulin
$\gamma$ IP-10	interferon- $\gamma$ -inducible CXCL10-like protein
$\mu$ M	micromolar
AGD	amoebic gill disease
AGD <sup>+</sup> L	lesion gill tissue from amoebic gill disease-affected fish
AGD <sup>+</sup> NL	non-lesion gill tissue from amoebic gill disease-affected fish
AGD <sup>-</sup> NL	non-lesion gill tissue from amoebic gill disease-naive fish
aRNA	amplified RNA
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium
BI	Bayesian inference
bp	base pairs
CCGD	clonal cultured gill derived
cDNA	complimentary DNA
C <sub>T</sub>	threshold cycle
CTL	cytotoxic T lymphocyte
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleotide triphosphate
DRTP	differentially-regulated trout protein
dTTP	deoxythymidine triphosphate
DTT	dithiothreitol
EF1 $\alpha$	elongation factor-1 $\alpha$
FasL	Fas ligand
FERR	ferritin
GTR	general time reversible evolutionary model
HKL	head kidney leucocytes
HKY	Hasegawa, Kishino and Yano evolutionary model
I $\kappa$ B	inhibitor of nuclear factor- $\kappa$ B
IFAT	immuno-fluorescent antibody test
IDO	indoleamine 2, 3-dioxygenase
IFN- $\alpha$	interferon- $\alpha$
IFN- $\gamma$	interferon- $\gamma$
IL-1 $\beta$	interleukin-1 $\beta$
IL-8	interleukin-8
IL-10	interleukin-10
iNOS	inducible nitric oxide synthase
IPTG	isopropyl- $\beta$ -D-thiogalactopyranoside
IRF-1	interferon regulatory factor-1
IRF-2	interferon regulatory factor-2
IRF-8	interferon regulatory factor-8
ISH	<i>in situ</i> hybridisation
ISRE	interferon-stimulated response element
ITS	internal transcribed spacer

## List of abbreviations

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JAK	Janus kinase
kDa	kilodalton
L-15	Lebovit's media
LB	Luria broth
LC	liquid chromatography
LPS	lipopolysaccharide
<i>M</i>	gene expression stability measure
M	molar
MHC I	major histocompatibility complex class I
MHC II	major histocompatibility complex class II
ML	maximum likelihood
mM	millimolar
MP	maximum parsimony
mRNA	messenger RNA
MS	mass spectrometry
MW	molecular weight
NCGD	non-cultured gill derived
NF- $\kappa$ B	nuclear factor- $\kappa$ B
NK	natural killer cell
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD1L	programmed death protein 1 ligand
PD2L	programmed death protein 2 ligand
PHA	phytohaemagglutinin
PLOs	<i>Perkinsela amoebae</i> -like organisms
PRRs	pattern recognition receptors
PSRF	potential scale reduction factor
qRT-PCR	quantitative real-time polymerase chain reaction
rIFN- $\gamma$	recombinant interferon- $\gamma$
RNA	ribonucleic acid
RPL2	RNA polymerase 2
rRNA	ribosomal RNA
RT	room temperature
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
STAT	signal transducers and activators of transcription
TAP	transport-associated with antigen processing protein
TBR	tree-bisection-reconnection
TGF- $\beta$	transforming growth factor- $\beta$
TNF- $\alpha$	tumour necrosis factor- $\alpha$
TRAIL	tumour necrosis (TNF)-related apoptosis-inducing ligand
TrN	Tamura-Nei evolutionary model
UTR	untranslated region

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# **1 GENERAL INTRODUCTION**

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## 1.1 Amoebic gill disease

Since the late 1970s, salmonid aquaculture has grown into a global industry and is now wide-spread throughout temperate regions (FAO, 2008). In the early 1980s, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) were introduced and farmed in marine sea-cages in the temperate coastal waters off southern Tasmania, Australia. At the onset of the first summer, the fish held in sea-cages experienced epizootics (Munday, 1986; Munday et al., 1990) with mortalities estimated at 2% of fish stocked per day (Munday et al., 1993). Clinical signs of disease included lethargy and behaviour associated with respiratory distress and eventual death (Munday et al., 1990). Gross signs of disease were restricted to the gills where amoebic branchialitis manifested as multifocal lesions that appeared as pale gill tissue (Munday et al., 1990; Rodger and McArdle, 1996). Histologically, the white gill patches were focal and multi-focal regions of epithelial-like cell hyperplasia (Munday et al., 1990). Progression of cellular hyperplasia led to the loss of gill architecture as the secondary lamellae fused and formed gill lesions (Adams and Nowak, 2003). The gills of affected fish were colonised by an ectoparasitic, homogenous population of small amoebae that were consistently associated with gill lesions, hence Munday (1993) coined the generic name amoebic gill disease (AGD).

Concurrent with the cases in Tasmania, AGD was also described in sea-cage cultured Coho salmon (*Oncorhynchus kisutch*) in Washington State, USA (Kent et al., 1988). Subsequently cases have been documented in a variety of fish species and geographical locations (Table 1.1). Whilst emerging as a globally significant disease, AGD continues to be the most significant condition to affect the culture of Atlantic

salmon in Tasmania with management of AGD amounting to \$15-20 million per annum (equivalent to 10 to 20% of production costs) (Vincent, 2008).

Table 1.1 Fish species and geographical locations where AGD has been reported.

Species	Location	Reference
Atlantic salmon ( <i>Salmo salar</i> )	Tasmania, Australia Spain Ireland  Chile  France	(Munday et al., 1990) (Munday et al., 2001) <sup>a</sup> (Palmer et al., 1997; Rodger and McArdle, 1996) (Clark and Nowak, 1999; Howard and Carson, 1993b) <sup>a</sup> (Clark and Nowak, 1999; Findlay et al., 1995) <sup>a</sup>
Brown trout ( <i>Salmo trutta</i> )	France	(Findlay et al., 1995) <sup>a</sup>
Chinook salmon ( <i>Oncorhynchus tshawytscha</i> )	New Zealand	(Howard and Carson, 1993b) <sup>a</sup>
Coho salmon ( <i>Oncorhynchus kisutch</i> )	Washington State, U.S.A	(Kent et al., 1988)
European seabass ( <i>Dicentrarchus labrax</i> )	Mediterranean	(Dyková et al., 2000)
Mediterranean seabream ( <i>Sparus aurata</i> )	Mediterranean	(Athanasopoulou et al., 2002) <sup>a</sup>
Rainbow trout ( <i>Oncorhynchus mykiss</i> )	Tasmania France	(Munday et al., 1990) (Findlay et al., 1995) <sup>a</sup>
Sharpsnout seabream ( <i>Diplodus puntazzo</i> )	Mediterranean	(Dyková et al., 2000)
Turbot ( <i>Psetta maxima</i> )	Spain	(Dyková et al., 1995; Dyková et al., 1998)

<sup>a</sup> Reported but AGD diagnosis not confirmed

Over 20 years of AGD research has resulted in significant progress in regards to characterising the pathological changes to AGD-affected gill tissue (Adams et al., 2004; Adams and Nowak, 2001; Adams and Nowak, 2003; Adams and Nowak, 2004b; Clark and Nowak, 1999; Clark et al., 2003; Dyková et al., 1995; Kent et al., 1988; Nowak and Lucas, 1997; Rodger and McArdle, 1996), the development of disease management strategies (Clark et al., 2003; Green et al., 2005; Harris et al., 2004; Parsons et al., 2001; Powell and Clark, 2004; Roberts and Powell, 2003a; Roberts and Powell, 2003b), the physiological response of the host (Leef et al., 2005a; Leef et al., 2005b; Powell and Nowak, 2003; Powell et al., 2002) and epidemiology of AGD (Clark and Nowak, 1999; Crosbie et al., 2003; Douglas-Helders et al., 2002; Douglas-Helders et al., 2005; Douglas-Helders et al., 2000; Douglas-Helders et al., 2003b; Douglas-Helders et al., 2001b). In addition, two other

research topics have received considerable attention and yet continue to generate more questions than answers; the aetiology of AGD and the host immune response to infection. These research topics are not mutually exclusive as it is important to know which pathogen(s) causes disease in order to characterise the specific host response. Since the aim is to address both of the research topics within this thesis they will be introduced in detail below.

## **1.2 Aetiology of AGD**

Amoeboid organisms are usually amorphous and this makes their taxonomic identification challenging. Hence, when identifying the amoebae intimately associated with AGD lesions the most prominent morphological feature used for their taxonomic classification was the presence of one or more perinuclear, eukaryotic endosymbiont referred to as parasomes or “Nebenkörper” (Schaudinn, 1896). This morphological feature restricted their taxonomic classification to three genera of marine amoebae, the *Paramoeba*, *Neoparamoeba* and *Janickina* (Page, 1983). Upon further examination, amoebae associated with AGD were revealed to lack the well organised cell-surface structures of many marine amoebae, including genus *Paramoeba* (Dyková et al., 1995; Kent et al., 1988; Page, 1987; Roubal et al., 1989). This led to the classification of AGD-associated amoebae as being members of the genus *Neoparamoeba* (Dyková et al., 2000).

The *Neoparamoeba* are a member of the Dactylopodida family and are ubiquitous in marine environments (Page, 1973; Page, 1983). Their trophozoites are small, lobose and form dactylopodiate subpseudopodia in their locomotive form. The ‘parasomes’

of *Neoparamoeba* are kinetoplastid-like, aflagellate, usually binucleated eukaryotic cells with a single, giant kinetoplastid-mitochondrion and are phylogenetically related to parasitic marine protozoans of the *Ichthyobodo* genus (Dyková et al., 2003; Moreira et al., 2004). Morphological similarities between *Neoparamoeba* “parasomes” and *Perkinsiella amoebae*, an endosymbiont of the *Janickina* amoebae (Hollande, 1980) led to *Neoparamoeba* “parasomes” being named *Perkinsiella amoebae*-like organisms (PLOs) (Dyková et al., 2003).

Initially, *N. pemaquidensis* was considered the single aetiological agent of AGD in marine fish (Kent et al., 1988; Roubal et al., 1989) and this conclusion was based upon morphological features, primarily the presence of one or more ‘parasomes’ and the lack of surface scales (Kent et al., 1988; Roubal et al., 1989). However, these morphological characteristics do not discriminate between members of the *Neoparamoeba* genus (Dyková et al., 2000; Dyková et al., 2005b). Therefore, the initial interpretation of *N. pemaquidensis* as the aetiological agent of AGD was presumptive. Immunological detection of *Neoparamoeba* using anti-*N. pemaquidensis* antiserum was successful and supported the role of *N. pemaquidensis* in AGD aetiology (Howard and Carson, 1993b). Based on these data, *N. pemaquidensis* was widely reported as the sole aetiological agent of AGD in Atlantic salmon (Adams and Nowak, 2001; Bowman and Nowak, 2004; Clark et al., 2003; Douglas-Helders et al., 2001a; Douglas-Helders et al., 2002; Douglas-Helders et al., 2003a; Douglas-Helders et al., 2005; Douglas-Helders et al., 2000; Douglas-Helders et al., 2003b; Douglas-Helders et al., 2001b; Douglas-Helders et al., 2003c; Munday et al., 2001; Powell and Clark, 2003; Powell and Clark, 2004; Tan et al., 2002; Wong

et al., 2004). However the anti-*N. pemaquidensis* antiserum was later shown to bind non-specifically to other marine amoebae (Howard, 2001; Morrison et al., 2005) and therefore, once again the interpretation of *N. pemaquidensis* as the aetiological agent of AGD was presumptive.

Recently, a new species of *Neoparamoeba*, *N. branchiphila* was cultured from the gills of AGD-affected fish (Dyková et al., 2005b). This amoeba was characterised using a combination of morphological and molecular phylogenetic analyses inferred from 18S rRNA gene sequences (Dyková et al., 2005b; Fiala and Dyková, 2003) and were clearly differentiated from *N. pemaquidensis* and *N. aestuarina* (Page, 1970). These data also resolved inter-specific relationships within the *Neoparamoeba* group (Dyková et al., 2005b; Fiala and Dyková, 2003; Mullen et al., 2005), with the segregation of phylogenetic lineages suggesting that AGD may be a disease of mixed aetiology. The possibility that more than one species of *Neoparamoeba* may elicit AGD led to the development of species-specific diagnostic tools, based upon 18S rRNA gene amplification by PCR to study disease aetiology where *Neoparamoeba* were the presumptive pathogens (Dyková et al., 2005b; Fiala and Dyková, 2003; Mullen et al., 2005; Wong et al., 2004). Indeed, PCR amplification of DNA isolated from amoebae cultured from gill tissues of AGD-affected fish was consistent with the proposition that both *N. pemaquidensis* and *N. branchiphila* may be associated with AGD (Dyková et al., 2005b; Wong et al., 2004).

There is now strong support for the primary pathogenic role of *Neoparamoeba* spp. in eliciting AGD (Adams and Nowak, 2004a). However, attempts to determine the pathogenicity of either *N. pemaquidensis* or *N. branchiphila* by re-infecting fish

using clonal cultured, gill-derived (CCGD) strains of either species have been universally unsuccessful (Howard and Carson, 1993b; Kent et al., 1988; Morrison et al., 2005; Vincent et al., 2007). Therefore, while the presence of *N. pemaquidensis* and *N. branchiphila* on the gills of AGD-affected fish is unequivocal (Dyková et al., 2005b; Fiala and Dyková, 2003), their role in AGD aetiology is yet to be confirmed formally. Consequently, virulent amoebae associated with AGD are often referred to as *Neoparamoeba* spp. (for example Attard et al., 2006; Gross et al., 2006; Morrison et al., 2005).

### **1.3 Host immune response to *Neoparamoeba* spp.**

The outer epithelial layers of a fish constitute the first line of defence against ectoparasites, providing the host with a layer of physical protection as well as tissue capable of secreting an array of compounds that initiate and modulate the immune response (Gonzalez et al., 2007a; Gonzalez et al., 2007b; Lindenstrom et al., 2003; Lindenstrom et al., 2004; Singh et al., 2004a; Singh et al., 2004b). Like most vertebrates, fish employ a repertoire of host defence mechanisms against ectoparasites that work synergistically to develop a cell-mediated inflammatory response (Buchmann et al., 2001; Chin and Woo, 2005; Cross and Matthews, 1993; Hines and Spira, 1974a) and acquired resistance to infection (Bakke et al., 1991; Chin et al., 2004; Clark et al., 1987; Hines and Spira, 1974b; Lindenstrom and Buchmann, 2000; Singh and Buchmann, 2001).

Only the gills appear to be affected by AGD with no definitive evidence of histological changes (Dyková et al., 1995; Kent et al., 1988; Rodger and McArdle, 1996) or immunological response (Bridle et al., 2006a; Bridle et al., 2006b) observed

in other organs. The most prominent cellular response in AGD lesions is the hyperplasia of epithelial-like cells at the site of *Neoparamoeba* spp. attachment (Adams and Nowak, 2001; Adams and Nowak, 2003; Dyková et al., 1995; Kent et al., 1988; Munday et al., 1993; Rodger and McArdle, 1996; Roubal et al., 1989). Despite the hyperplastic cellular response within AGD lesions there is only a modest immune cell-like response. For instance, whilst leucocytes have been observed to migrate toward AGD lesions, they primarily remain in the central venous sinus, adjacent to lesions and seldom are they seen within the lesions themselves (Adams and Nowak, 2001; Adams and Nowak, 2003).

The innate immune response has been suggested to be an important factor in conferring protection to AGD-affected fish (Bridle et al., 2003; Findlay and Munday, 1998; Zilberg and Munday, 2000). Based on this premise, immunostimulants were used to elicit an innate immune response in AGD-affected fish (Bridle et al., 2003; Bridle et al., 2005; Findlay and Munday, 2000; Findlay et al., 2000).

Oligodeoxynucleotides containing cytosine-phosphodiester-guanine motifs (CpGs) can stimulate a systemic cell-mediated immune response in fish (Jorgensen et al., 2001a; Jorgensen et al., 2001b) and preliminary evidence suggested that they may increase the short-term resistance to AGD (Bridle et al., 2003 and Morrison, unpublished). However, whilst these results were promising, immunostimulants have not been shown to protect fish from AGD (Bridle et al., 2003).

Table 1.2. Transcriptional response of immunoregulatory genes in amoebic gill disease-affected salmonids

Gene	Description	Expression	Reference
Interleukin-1 $\beta$ (IL-1 $\beta$ )	Proinflammatory cytokine	Up	(Bridle et al., 2006a; Bridle et al., 2006b; Morrison et al., 2007)
Interferon- $\gamma$ (IFN- $\gamma$ )	Type II immunoregulatory cytokine	Stable	(Morrison et al., 2007)
Tumour necrosis factor- $\alpha$ (TNF- $\alpha$ )	Proinflammatory cytokine	Stable	(Bridle et al., 2006b; Morrison et al., 2007)
Transforming growth factor- $\beta$ (TGF- $\beta$ )	Growth factor	Stable	(Bridle et al., 2006b)
inducible nitric oxide synthase (iNOS)	Inducible immunoregulatory enzyme	Stable	(Bridle et al., 2006b; Morrison et al., 2007)
Serum amyloid A (SAA)	Acute phase protein	Stable	(Bridle et al., 2006b)
Serum amyloid P-like protein, pentraxin (SAP)	Acute phase protein	Stable	(Bridle et al., 2006b)
Major histocompatibility complex class II- $\beta$ (MHC II $\beta$ )	Presentation of exogenous antigens	Stable	(Bridle et al., 2006b)
T cell receptor- $\beta$	T cell receptor molecule	Stable	(Bridle et al., 2006b)



The adaptive immune response in AGD-affected Atlantic salmon has also been investigated (Akhlaghi et al., 1996; Findlay and Munday, 1998; Gross et al., 2004a; Gross et al., 2004b; Gross et al., 2005; Vincent et al., 2006). Promisingly, antibodies were produced against virulent *Neoparamoeba* spp. in AGD-affected Atlantic salmon reared in laboratory conditions (Vincent et al., 2006) and in Atlantic salmon held under sea-caged culture conditions where AGD is endemic (Gross et al., 2004a). However, in these cases the presence of antibodies was not associated with protective immunity to *Neoparamoeba* spp. (Gross et al., 2004a; Vincent et al., 2006).

The host immune response to *Neoparamoeba* spp. has also been investigated at the molecular level using a gene expression approach. *Neoparamoeba* spp. induced interleukin-1 (IL-1 $\beta$ ) mRNA expression in AGD-affected gill tissues (Bridle et al., 2006a; Bridle et al., 2006b). IL-1 $\beta$  is a pro-inflammatory cytokine that mediates the fish proinflammatory immune response to a variety of fish pathogens (Bridle et al., 2006a; Bridle et al., 2006b; Cuesta et al., 2006; Lindstrom et al., 2003; Morrison et al., 2007; Singh et al., 2004a). Interestingly, an up-regulation in IL-1 $\beta$  occurs without change in the expression of either tumour necrosis factor- $\alpha$  [TNF- $\alpha$ , (Morrison et al., 2007)] or inducible nitric oxide synthase [iNOS, (Bridle et al., 2006b; Morrison et al., 2007)], genes typically activated in response to IL-1 (Laing et al., 2001). In fact, many genes that are important in the regulation of a host immune response were not transcriptionally activated in AGD-affected salmonid gill tissues (Table 1.2). On a more wide-spread scale, 190 unique transcripts were identified as being up or down-regulated in AGD-affected gill tissues (Morrison et al., 2006a). While Morrison et al. (2006a) found no definitive evidence of an immunological response to AGD they did demonstrate that a disruption in transcription of the p53 tumour suppressor protein may mediate the hyperproliferative response observed in AGD lesions. These data

also supported the opinion that aspects of the innate and adaptive immune response are inactive during the host response to AGD.

#### **1.4 Aims and thesis outline**

AGD has been the subject of considerable research effort, and yet it still remains unclear which members of the morphologically similar *Neoparamoeba* group play a role in AGD aetiology. Elucidating AGD aetiology is critical for the development of specific diagnostic assays for AGD as well as ensuring that potential vaccine candidate antigens are targeted toward the correct species. Therefore a series of studies were conducted to:

1. Determine the aetiological agent(s) of AGD affecting Atlantic salmon cultured in marine sea-cages in Tasmania, Australia.
2. Determine the aetiological agent(s) of AGD globally.
3. Determine the phylogenetic relationship between the aetiological agent(s) of AGD and other members of the *Neoparamoeba*.
4. Develop a diagnostic assay for the aetiological agent(s) of AGD

The first and second aims were accomplished by identifying the aetiological agent(s) of AGD *in situ* in order to corroborate the link between *Neoparamoeba* species and their association with AGD lesions. The use of ribosomal RNA (rRNA)-targeted nucleic acid probes in differentiating specific groups or species of bacteria in

microbial ecological studies is well established (see review Amann and Ludwig, 2000). *In situ* hybridisation using oligonucleotides that target rRNA from pathogenic amoebae has been utilised successfully for both clinical and environmental samples (Dyková et al., 2005c; Grimm et al., 2001; Kleeman et al., 2002; Stothard et al., 1999). Here species-specific oligonucleotide probes were developed that target rRNA and these were used in a series of *in situ* hybridisation experiments to identify *Neoparamoeba* species associated with AGD gill lesions.

An understanding of the phylogenetic relationship between *Neoparamoeba* and other members of the Dactylopodida is developing rapidly with the discovery of new species and strains (Dyková et al., 2005b; Fiala and Dyková, 2003; Mullen et al., 2005; Peglar et al., 2003). In these studies, full-length 18S (small subunit) rRNA gene sequences from amoebae were used in conjunction with morphology to characterise *Neoparamoeba* species. These data were useful for assigning amoebae to their correct taxonomic groups and the development of diagnostic tools (Dyková et al., 2005b; Elliott et al., 2001; Mullen et al., 2005; Wong et al., 2004). To address the third aim, phylogenetic data collected within this thesis were integrated within the current *Neoparamoeba* phylogeny (Dyková et al., 2005b) to characterise strains of the virulent *Neoparamoeba* spp. and compare their phylogenetic relationship with other members of the *Neoparamoeba*.

In aquaculture operations affected by AGD, the prevalence and density of gross AGD lesions is monitored by commercial marine Atlantic salmon (*Salmo salar* L.) growers in order to estimate the severity of AGD and manage treatment (Adams et

al., 2004; Clark and Nowak, 1999; Clark et al., 2003). Since other agents can elicit gill lesions macroscopically indistinguishable from AGD-related lesions (Adams et al., 2004; Clark et al., 1997) all farm-based assessments of AGD are presumptive. Previously, 18S rRNA gene amplification by PCR was used to study disease aetiology where *Neoparamoeba* were the presumptive pathogens (Dyková et al., 2005b; Fiala and Dyková, 2003; Mullen et al., 2005; Wong et al., 2004). To address the fourth aim, a similar approach was pursued here, whereby 18S rRNA gene data collected over the course of this thesis were used to design a PCR to amplify the 18S rRNA gene of *Neoparamoeba* spp. within the gill tissues of AGD-affected Atlantic salmon as a complementary method of diagnosing AGD in the field.

Atlantic salmon are highly susceptible to AGD which, if left untreated, is a significant cause of mortality in fish reared under farm conditions (Munday et al., 1993; Munday et al., 2001). Based on studies to date there is little, if any, definitive evidence that AGD-affected fish have the capacity to develop innate (Bridle et al., 2006a; Bridle et al., 2006b) or acquired (Akhlaghi et al., 1996; Findlay and Munday, 1998; Gross et al., 2006; Gross et al., 2004b; Morrison et al., 2006a; Vincent et al., 2006) resistance that minimises the impact of ectoparasitosis by *Neoparamoeba* spp. Understanding the biological mechanisms that restrict the host response to AGD is critical for developing AGD treatments. Therefore, as a component of this thesis a series of studies will be conducted to:

5. Determine why Atlantic salmon are highly susceptible to AGD; and,

6. Determine whether their susceptibility is linked to the aberrant innate and adaptive immune responses.

To address the fifth and sixth aims the genomics resources developed by the Genomic Research on Atlantic salmon Project (GRASP) (Rise et al., 2004b) including a cDNA microarray (von Schalburg et al., 2005) were utilised to determine the biological mechanisms that mediate the susceptibility of Atlantic salmon to AGD. The use of functional genomics (i.e. gene expression), in particular the use of cDNA microarrays, has been employed successfully to elucidate the cellular pathways that contribute to a variety of physiological and pathogenic states in salmonids (Ewart et al., 2005; Krasnov et al., 2005; MacKenzie et al., 2006; Morrison et al., 2006a; Purcell et al., 2006; Rise et al., 2006; Rise et al., 2004a; von Schalburg et al., 2005; Vuori et al., 2006). In fact, a recent study utilised a cDNA microarray to profile the transcriptional response within AGD-affected gill tissues at the early onset of disease (0-14 days post-exposure to *Neoparamoeba* spp.) (Morrison et al., 2006a). Whilst Morrison et al. (2006a) identified molecular mechanisms that maintain AGD lesions in a hyperplastic cell state there was no definitive evidence of a disruption in immunoregulatory genes. Over progressive studies it became apparent that the transcriptional response of individual genes was restricted to AGD lesions (Bridle et al., 2006a; Morrison et al., 2006a; Morrison et al., 2007). Therefore, when examining AGD-affected gill tissue, a failure to specifically target AGD lesions may have masked a specific transcriptional response (Morrison et al., 2006a). Therefore the hypothesis that the transcriptional response may be restricted to the micro-environment within AGD lesions was addressed.

Thus, cDNA microarray analyses were used to determine the transcriptome profiles from AGD lesions in comparison to “normal” gill tissues from the same arch of AGD-affected fish or from AGD-naïve fish.

Transcriptome profiling the gills of AGD-affected fish identified a disruption of MHC antigen processing and presentation pathways within AGD lesions (Chapter 6). Since this was associated with a down-regulation in the type II cytokine, IFN- $\gamma$  it was proposed this immunoregulatory cytokine may mediate the disruption in antigen processing and presentation within AGD-lesions. Therefore a final series of experiments, described in Chapter 7, were performed to examine the role of aberrant IFN- $\gamma$  signalling in the transcriptional response of Atlantic salmon gill tissues to AGD. More specifically, the aim of this study was to:

7. Determine whether stimulation of AGD-affected Atlantic salmon gills with IFN- $\gamma$  restores normal expression of genes associated with antigen processing and presentation.

In a final chapter (Chapter 8), the most significant findings from the data chapters are synthesised and placed in context with the current scientific literature. Moreover, the potential significance of the knowledge generated for the improved diagnosis and treatment of AGD in marine fish aquaculture operations globally are described.

## **1.5 Explanatory notes regarding thesis structure**

This thesis is structured as a series of chapters that are either published or are manuscripts to be submitted for publication. As a consequence, some textual overlap occurs between chapters. The general introduction to this thesis is written as a review of the relevant literature at the commencement of this PhD project, therefore some literature is omitted but will be considered in later chapters. For instance, the taxonomic nomenclature used in the general introduction is different from the later chapters due to the inclusion of additional literature (for example: Dyková et al. (2008) recently renamed *Perkinsiella* to *Perkinsela*). Relevant research that was collected after the publication of these research chapters will be considered in Chapter 8. The referencing style of the journal Molecular Immunology has been adopted for this thesis and a single bibliography is presented at the end of the thesis.

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**2    *NEOPARAMOEBA PERURANS* N. SP., AN AGENT OF AMOEBIC GILL  
DISEASE OF ATLANTIC SALMON (*SALMO SALAR*)**

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## 2.1 Abstract

Amoebic gill disease (AGD) is a potentially fatal disease of some marine fish. Two amphizoic amoebae *Neoparamoeba pemaquidensis* and *N. branchiphila* have been cultured from AGD-affected fish yet it is not known if one or both are aetiological agents. Here, we PCR amplified the 18S rRNA gene of non-cultured, gill-derived (NCGD) amoebae from AGD-affected Atlantic salmon (*Salmo salar*) using *N. pemaquidensis* and *N. branchiphila*-specific oligonucleotides. Variability in PCR amplification led to comparisons of 18S (small subunit) and 28S (large subunit) rRNA gene sequences from NCGD and clonal cultured, gill-derived (CCGD) *N. pemaquidensis* and *N. branchiphila*. Phylogenetic analyses inferred from either 18S or 28S rRNA gene sequences unambiguously segregated a lineage consisting of NCGD amoebae from other members of the *Neoparamoeba* genus. Species-specific oligonucleotide probes that hybridise 18S rRNA were designed, validated and used to probe gill tissue from AGD-affected Atlantic salmon. The NCGD amoebae-specific probe bound AGD-associated amoebae while neither *N. pemaquidensis* nor *N. branchiphila* were associated with AGD-lesions. Together, these data indicate that NCGD amoebae are a new species, designated *N. perurans* n. sp. and this is the predominant aetiological agent of AGD of Atlantic salmon cultured in Tasmania, Australia.

## 2.2 Introduction

Amoebae of the Vexilliferidae and Paramoebidae families are ubiquitous within marine and estuarine environments (Page, 1973; Page, 1987). Phylogenetically these families converge to create the so-called “PV lineage” (Peglar et al., 2003).

*Neoparamoeba* spp. (Lobosea) belong to the Vexilliferidae family and are small, lobose amoebae that form dactylopodiate subpseudopodia in their locomotive form. They lack the well organised cell-surface structures of other Vexilliferids such as hexagonal glycostyles (*Vexillifera*) or surface scales (*Korotnevella*) and possess one or more intracellular perinuclear bodies, known as ‘parasomes’. These ‘parasomes’ are described as *Perkinsiella amoebae*-like organisms (PLOs) and are eukaryotic endosymbionts, phylogenetically related to flagellated, parasitic marine protozoans of the *Ichthyobodo* genus (Dyková et al., 2003).

*Neoparamoeba* is an ecologically important group since it contains amoebae that are reportedly amphizoic. Initially, *N. pemaquidensis* was considered the single aetiological agent of amoebic gill disease (AGD) in Atlantic salmon (*Salmo salar*, ) (Kent et al., 1988; Roubal et al., 1989) and this conclusion was based upon morphological features, primarily the presence of one or more ‘parasomes’ and the lack of surface scales (Kent et al., 1988; Roubal et al., 1989). While morphological characteristics distinguish *Neoparamoeba* from other Vexilliferids, attempts to demarcate members of the *Neoparamoeba* genus using morphological characteristics alone have been unsuccessful (Dyková et al., 2000; Dyková et al., 2005b). Therefore, the initial interpretation of *N. pemaquidensis* as the aetiological agent of AGD was presumptive. Immunological detection of *Neoparamoeba* using anti-*N.*

*pemaquidensis* antiserum was successful and supported the role of *N. pemaquidensis* in AGD aetiology (Howard and Carson, 1993b). However the anti-*N. pemaquidensis* antiserum was later shown to bind non-specifically to other marine amoebae (Morrison et al., 2005).

Recently, a new species of *Neoparamoeba*, *N. branchiphila* was cultured from the gills of AGD-affected fish (Dyková et al., 2005b). This amoeba was characterised using a combination of morphological and molecular phylogenetic analyses inferred from 18S (small subunit) rRNA gene sequences (Dyková et al., 2005b; Fiala and Dyková, 2003) and were clearly differentiated from *N. pemaquidensis* and *N. aestuarina* (Page, 1970). These data also resolved inter-specific relationships within the *Neoparamoeba* group (Dyková et al., 2005b; Fiala and Dyková, 2003; Mullen et al., 2005), with the segregation of phylogenetic lineages suggesting that AGD may be a disease of mixed aetiology. This led to the development of species-specific diagnostic tools, based upon 18S rRNA gene amplification by PCR to study disease aetiology where *Neoparamoeba* were the presumptive pathogens (Dyková et al., 2005b; Fiala and Dyková, 2003; Mullen et al., 2005; Wong et al., 2004). Indeed, PCR amplification of DNA isolated from amoebae cultured from gill tissues of AGD-affected fish was consistent with the proposition that both *N. pemaquidensis* and *N. branchiphila* may be associated with AGD (Dyková et al., 2005b; Wong et al., 2004). However, attempts to determine the pathogenicity of either species by re-infecting fish using clonal cultured, gill-derived (CCGD) strains have been universally unsuccessful (Howard and Carson, 1993b; Kent et al., 1988; Morrison et al., 2005; Vincent et al., 2007). While the presence of *N. pemaquidensis* and *N.*

*branchiphila* on the gills of AGD-affected fish is unequivocal (Dyková et al., 2005b; Fiala and Dyková, 2003; Wong et al., 2004), their role in AGD aetiology is yet to be confirmed formally.

Here, we used a PCR-based approach to examine the abundance of *N. pemaquidensis* and *N. branchiphila* in amoebae preparations directly isolated from the gills of AGD-affected Atlantic salmon. Persistent variability in PCR amplification led to phylogenetic analyses of 18S and 28S rRNA gene sequences obtained from non-cultured gill-derived (NCGD) amoebae. Evidence is presented to support the creation of a new phylogenetically distinct lineage of *Neoparamoeba*, exclusive to NCGD amoebae. Using *in situ* hybridisation (ISH) with *Neoparamoeba* species-specific probes we verified that the only detectable amoebae directly associated with AGD lesions in Atlantic salmon in Tasmania all belonged to this new phylogenetic lineage. This undermines the putative role of *N. pemaquidensis* and *N. branchiphila* in AGD.

## **2.3 Materials and Methods**

### **2.3.1 Acquisition of clonal, cultured gill-derived (CCGD) and non-cultured gill-derived (NCGD) amoebae**

AGD-affected Atlantic salmon, *Salmo salar* were obtained from a recirculation tank-based population of fish (D1) maintained at the School of Aquaculture, University of Tasmania (Launceston, Tasmania, Australia). This recirculation tank was originally populated with AGD- affected Atlantic salmon from the Huon Estuary, Tasmania, Australia (Huon Aquaculture Company) in October, 2001 and the disease has been propagated by cohabitation with naïve fish. Farm-reared AGD-affected Atlantic

salmon were obtained from the Huon Estuary, Tasmania, Australia (Huon Aquaculture Company). Fish were anaesthetised (50 mg L<sup>-1</sup> Aqui-S NZ Ltd, Lower Hutt, New Zealand) and assessed for AGD lesions as previously described (Munday et al., 2001). Fish presumptively diagnosed with AGD were euthanized (100 mg L<sup>-1</sup> Aqui-S) and amoebae were directly isolated from gill tissues as previously described (Morrison et al., 2004) herein termed NCGD amoebae.

Clonal cultures of amoeba strains were obtained from a culture collection held at the School of Aquaculture, University of Tasmania (Table 2.1). Previous identification of strains was based on phylogenetic analyses and 18S rRNA gene-specific PCR (Dyková et al., 2005b; Wong et al., 2004). Amoebae culture and harvesting procedures followed those previously described (Dyková et al., 2000; Dyková et al., 2005b). Harvested and purified trophozoites were maintained for no longer than 30 min in phosphate buffered saline, pH 7.4 PBS) until further processing.

### **2.3.2 DNA extraction and assessment of NCGD amoebae using 18S rRNA gene PCR**

Aliquots of between  $1-5 \times 10^5$  amoebae were centrifuged ( $10,000 \times g$ , 1 min) and the supernatant removed. The cell pellet was stored at -80°C or processed immediately. Genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen, Doncaster, Victoria, Australia) as per the manufacturer's instructions.

Table 2.1. Marine amoebae included in either phylogenetic analyses using partial sequences of the 28S rRNA gene or included in a *Neoparamoeba* tissue array.

Species	Culture ID <sup>a</sup>	Origin <sup>b</sup>	Accession Number <sup>c</sup>
<i>Neoparamoeba pemaquidensis</i>	PA027*	Dover, G	AF371967
	NP251002*	Infection Tank, University of Tasmania, G	AY714351
	WTUTS*	Infection Tank, University of Tasmania, G	AY714361
	GILLNOR1*	D'Entrecasteaux Channel, Bruny Island, G	AY714352
<i>Neoparamoeba branchiphila</i>	NRSS*	Infection Tank, University of Tasmania, G	AY714367
	ST4N*	Huon Estuary, Dover, G	AY714365
	SEDMH1*	Macquarie Harbour, S	AY714366
<i>Paraflabellula hoguae</i>	NETC3	Huon Estuary, Dover, N	AY277797

<sup>a</sup> Clonal culture identification tags from a collection held at the School of Aquaculture, University of Tasmania, Launceston, Tasmania, Australia.

<sup>b</sup> Region of Tasmania, Australia from which original isolates were derived and whether they were cultured from gill (G). sea-cage net (N) or sediment (S) samples

<sup>c</sup> GenBank accession numbers (<http://www.ncbi.nlm.nih.gov/>)

\*Amoebae included within the *Neoparamoeba* tissue array

The presence of *N. pemaquidensis* and *N. branchiphila* in NCGD amoebae primary isolates was confirmed with 18S rRNA gene PCR using species-specific oligonucleotides as previously described (Dyková et al., 2005b; Wong et al., 2004). Where amplification was unsuccessful, PCR was repeated using 35 cycles. PCR amplification efficiency of template DNA was assessed with universal 18S rRNA gene oligonucleotides [18e and 18i (Hillis and Dixon, 1991)] as previously described (Mullen et al., 2005). All PCR reactions were initiated with 20 ng of purified DNA template. Control templates included genomic DNA from Atlantic salmon, cultured *N. pemaquidensis* and *N. branchiphila* strains and no template controls. PCR reactions were electrophoresed through 1 to 2% agarose/TBE.

### 2.3.3 Amplification and sequencing of 18S and 28S rRNA

The PCR amplification of the full-length 18S rRNA gene from amoebae was performed as previously described (Dyková et al., 2005b). PCR amplification of a portion of the 28S rRNA gene from amoebae including divergent domains D1 to D3 and the conserved core region upstream of domain D3 was performed using universal oligonucleotides as previously described [28 F and 1438 R (Bergholtz et al., 2005)]. Amplification of the 28S rRNA gene was performed in volumes of 25 µL containing 1 U of Platinum *Taq* (Invitrogen, Mount Waverley, Victoria, Australia), 1 × Platinum *Taq* PCR buffer, 200 µmol of each deoxynucleotide triphosphate (dNTP; dATP, dCTP, dGTP, and dTTP), 1.5 mmol of MgCl<sub>2</sub> and 10 pmol of each oligonucleotide. PCR cycle conditions were 94°C for 3 min; 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min, for 30 cycles; and 72°C for 10 min. Control templates included genomic DNA from Atlantic salmon, cultured *N. pemaquidensis* and *N. branchiphila* strains and no template controls.

PCR products were ligated into pGEM-T easy plasmid vector according to manufacturer's instructions (Promega, Annandale, Australia). After transformation into *Escherichia coli* strain DH10β, positive clones were identified by blue-white colour selection (BlueTech, Mirador DNA Design, Montreal, Quebec, Canada) followed by PCR. Clones (herein termed molecular clones) were inoculated into Luria broth and plasmid DNA was purified (MiniPrep, Qiagen). Nucleotide sequencing was performed using either the DTCS Quick Start Dye Terminator Kit (Beckman Coulter, Fullerton, California, USA) or ABI Prism BigDye Terminator

(version 3.1) Cycle Sequencing Kit (Applied Biosystems (ABI), Scoresby, Victoria, Australia) following the manufacturer's instructions. Sequencing reactions were initiated using plasmid DNA template and the insert amplified using M13 forward or reverse oligonucleotides. Samples were analysed on a CEQ 8000 sequencer (Beckman Coulter) or ABI 3730xl DNA analyser (ABI). The 18S and 28S rRNA gene sequences analysed in this study were deposited in GenBank (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD) with the accession numbers shown in Fig. 2.3 and 2.4 respectively.

#### **2.3.4 Construction and support for *Neoparamoeba* phylogeny using the 18S and 28S rRNA genes**

The 18S rRNA gene sequences from seven molecular clones from five independent NCGD amoebae primary isolates, 36 strains of *Neoparamoeba* obtained from GenBank and an outgroup containing *Korotnevella stella* (GenBank accession number: AY183893), *Korotnevella hemistylolepis* (AY121850), *Vexillifera armata* (AY183891), *Vannella anglica* (AF099101) and *Vannella aberdonica* (AY121853) were aligned in ClustalX (Thompson et al., 1997) (gap opening/gap extension penalty = 8/2). The outgroup contained species that were morphologically distinct from *Neoparamoeba* species but closely-related based on phylogenetic analyses of the 18S rRNA gene (Fiala and Dyková, 2003; Peglar et al., 2003). The alignment was checked manually using BioEdit (Hall, 1999) and has been submitted to the European Molecular Biology Laboratory (EMBL)-Align database (accession number ALIGN\_001117). Genetic distances among 18S rRNA gene sequences were



calculated as mean character differences using PAUP\*, version 4.0b10 (Swofford, 2001). Phylogenetic tree searches using the aligned 18S rRNA gene sequences were conducted using three methods of analysis. Maximum parsimony analyses (MP) were performed in PAUP\*, version 4.0b10 using the heuristic search with tree-bisection-reconnection (TBR) branch swapping, the ACCTRAN option and 10 random-taxon addition iterations. Gaps were treated as missing data. Clade reliability was estimated using bootstrap resampling (Felsenstein, 1985) with 1000 replicates. The Akaike Information Criteria (AIC) test in Modeltest version 3.7 (Posada and Crandall, 1998) selected the general time reversible model of evolution with gamma distribution and a proportion of invariable sites (GTR +  $\Gamma$  +I) as the most appropriate likelihood model for further phylogenetic analyses. Tree searches by maximum likelihood (ML) analysis were performed in PAUP\*, version 4.0b10 using the heuristic process with TBR branch swapping. Estimates of the  $\alpha$ -parameter (0.5044), frequency of base proportions (A = 0.2858, C = 0.1600, G = 0.2228 and T = 0.3314), the substitution rate model matrix ([A-C] = 1.2941, [A-G] = 8.1120, [A-T] = 2.5793, [C-G] = 0.6096, [C-T] = 10.2555, and [G-T] = 1.0000) and proportion of invariable sites (0.2984) determined using the AIC test were fixed for the analysis. Clade reliability for the most parsimonious ML tree was estimated using bootstrap resampling with 1000 replicates generated by SEQBOOT in PHYLIP Version 3.66 (Felsenstein, 1989). ML analysis of the 1000 bootstrap replicates was performed with PHYML (Guindon and Gascuel, 2003) with the GTR +  $\Gamma$  +I model settings previously described. The ML tree obtained in PAUP\*4b10 was used as the starting tree. Bayesian phylogenetic inference (BI) was determined using Markov chain Monte Carlo (MCMC) analysis in MrBayes, version 3.1.2 (Huelsenbeck and

Ronquist, 2005; Ronquist and Huelsenbeck, 2003). The likelihood parameters that were set for BI were based on the GTR +  $\Gamma$  + I model. Three million generations of MCMC analysis were performed and trees were recorded every 100<sup>th</sup> generation. At this point, the standard deviation of split frequencies was < 0.01 and the potential scale reduction factor (PSRF) approached one. Consensus trees were generated using the 50% majority rule criterion on bootstrap replicate trees generated with MP and ML analyses and the final 75% of trees generated by BI.

Partial 28S rRNA gene sequences from six independent NCGD amoebae primary isolates, four strains of culture-purified *N. pemaquidensis*, three strains of *N. branchiphila* and *P. hoguae* (Table 2.1) were aligned in ClustalX (Thompson et al., 1997) (gap opening/gap extension penalty = 8/2). *P. hoguae* was selected as an outgroup as it is morphologically distinct from *Neoparamoeba* species but closely-related based on phylogenetic analysis of the 18S rRNA gene (Peglar et al., 2003). The partial 28S rRNA gene alignment was submitted to the EMBL-Align database (accession number ALIGN\_001118). Genetic distances among 28S rRNA gene sequences were calculated as described above. The same methods for phylogenetic analyses and the selection of likelihood models were used as described above for the 18S rRNA gene sequence alignment. The GTR +  $\Gamma$  + I likelihood model was selected for ML analysis and BI. Fixed parameter estimates using the AIC test for ML analysis were the  $\alpha$ -parameter ( 0.4756), frequency of base proportions (A = 0.3044, C = 0.1533, G = 0.2415 and T = 0.3008), the substitution rate model matrix ([A-C] = 0.6297, [A-G] = 10.6129, [A-T] = 3.6016, [C-G] = 1.2679, [C-T] = 13.6820, and [G-

TJ = 1.0000) and proportion of invariable sites (0.3685). BI using MCMC analysis was performed for  $5 \times 10^5$  generations and trees were recorded every 100<sup>th</sup> generation. At this point, the standard deviation of split frequencies was < 0.01 and PSRF approached one. Consensus trees were generated as described above for the 18S rRNA gene sequence alignment.

### **2.3.5 Design and validation of *Neoparamoeba* species-specific rRNA oligonucleotide probes and their application on AGD-affected Atlantic salmon gill tissue**

Oligonucleotide probes that hybridise to 18S rRNA from each clade of *Neoparamoeba* were designed where suitable base mis-matches were observed in aligned sequences. The probes were designed following established guidelines (Hugenholtz et al., 2001), synthesised and 5' end-labelled with digoxigenin (DIG) (Thermo Electron, Hamburg, Germany).

The specificity of each probe was validated using *in situ* hybridisation with representative *Neoparamoeba* strains (Table 2.1) that were embedded within a single paraffin block (termed a *Neoparamoeba* array). Approximately  $0.5-1 \times 10^6$  NCGD or CCGD amoebae were centrifuged at  $500 \times g$  for 5 min at 4°C and the supernatant removed. Further centrifugations were performed in a similar manner. Amoebae were resuspended in 30 mL of 1 × PBS, centrifuged and the supernatant removed. The cell pellet was resuspended in 10 mL of seawater Davidson's fixative (SWD)

and fixed for 30 min. Fixed amoebae were centrifuged and the supernatant removed. Amoebae were sequentially washed with 70% ethanol and 3× with PBS. The cells were finally resuspended in 100 µL of PBS, heated to 65°C for 5 min then mixed in a 0.2 mL PCR tube with 100 µL of premelted 2% agarose/PBS. Once set, the agarose blocks were dehydrated in 70% ethanol for 2 h and all strains were embedded within a single mould. Atlantic salmon skeletal muscle fixed in a similar manner was added to the array as a host rRNA control.

Gill tissues from AGD-affected Atlantic salmon from tank and field-based populations were obtained for *in situ* hybridisation. Atlantic salmon presumptively diagnosed with AGD were euthanized (100 mg L<sup>-1</sup> Aquí-S), the second left gill arch removed, placed in SWD for 24 h and processed for routine histology. Gills were sectioned (5 µm) and stained with haematoxylin and eosin (H & E) following routine histological procedures. After confirming that amoebae were attached to AGD lesions using light microscopy, tissues were further processed for *in situ* hybridisation.

Protocols were identical for hybridising the species-specific oligonucleotide probes to the rRNA within the *Neoparamoeba* array and AGD-affected gill tissues. Tissue sections (7 µm) were placed onto coated glass slides (Polysine, Menzel-Gläser, Braunschweig, Germany) and dried overnight at 37°C. Unless specified, all washes were performed at room temperature (RT). Sections were dewaxed, rehydrated and

sequentially washed for 2× 5 min with diethyl pyrocarbonate (Sigma-Aldrich, Castle Hill, New South Wales, Australia)-treated PBS (DEPC-PBS), 10 min with DEPC-PBS containing 100 mM glycine, 15 min with DEPC-PBS containing 0.3% Triton X-100 (Sigma-Aldrich) and 2× 5 min with DEPC-PBS. Sections were then permeabilised with 5 µg mL<sup>-1</sup> RNase-free proteinase K (Amresco, Solon, Ohio, U.S.A.) at 37°C for 30 min, post-fixed for 5 min in DEPC-PBS containing 4% paraformaldehyde (ProSciTech, Townsville, Queensland, Australia) at 4°C then washed in DEPC-PBS for 2× 5 min. Sections were acetylated in 0.1 M triethanolamine (Sigma-Aldrich) buffer, pH 8.0, containing 0.25% (v/v) acetic anhydride (Fluka, Castle Hill, New South Wales, Australia) for 10 min on a rocking platform. Sections were then overlaid with 80 µL prehybridisation buffer [2× saline sodium citrate (SSC), 1× Denhardt's solution, 10% dextran sulphate (Sigma-Aldrich), 50 mM phosphate buffer, pH 7.0, 50 mM DDT, 500 µg mL<sup>-1</sup> denatured and sheared cod DNA and a volume of deionised formamide (dF) (Sigma-Aldrich) specific for each probe (18S universal probe 61% dF; *N. pemaquidensis* probe 46% dF; *N. branchiphila* probe 37% dF and NCGD amoebae probe 56% dF)], a coverslip was added and slides were incubated in a humid chamber at 37°C for 2 h. Coverslips were removed by immersing sections in 2× SSC for 5 min. Sections were then overlaid with 80 µL hybridisation buffer with a probe (prehybridisation buffer and 4 ng µL<sup>-1</sup> probe) or without a probe (no probe controls), a coverslip was added and slides were incubated in a humid chamber at 37°C for 17 h. Coverslips were again removed in 2× SSC then the slides were sequentially washed on a shaking platform at 37°C in 2× SSC for 2× 15 min, 1× SSC for 2× 15 min and 0.25× SSC for 2× 15 min. DIG-labelled probe detection was performed using a BCIP/NBT immunological

method. Slides were washed on a shaking platform in tris buffered saline (TBS) (100 mM Tris-HCl, pH 7.5 and 150 mM NaCl) for 20 min. Sections were then covered with blocking solution (TBS containing 0.1% Triton X-100 and 2% normal sheep serum) and incubated for 30 min. The blocking solution was decanted and slides were covered with TBS containing 0.1% Triton X-100, 1% sheep serum and a 1:500 dilution of sheep anti-DIG-alkaline phosphatase (Roche, Kew, Victoria, Australia) for 2 h in a humid chamber. Slides were washed in TBS for 20 min on a shaking platform then incubated for 10 min with TBS-MgCl<sub>2</sub> (100 mM Tris-HCl, 100 mM NaCl and 50 mM MgCl<sub>2</sub>, pH 9.5). The TBS-MgCl<sub>2</sub> was decanted and sections were overlayed with a premixed BCIP/NBT solution (Sigma-Aldrich) and incubated for up to 2 h. The reaction was monitored under a light microscope and stopped by briefly washing slides in TE buffer (10 mM Tris-HCl, pH 8.1 and 1 mM EDTA). Sections were counterstained for 5 min in 0.1% nuclear fast red (Sigma-Aldrich), dehydrated and mounted (VectaMount™, Vector Laboratories, Burlingame, California, U.S.A).

## **2.4 Results**

### **2.4.1 Confirmation of the presence of *Neoparamoeba* species in Atlantic salmon NCGD amoebae primary isolates**

To determine whether *N. pemaquidensis* or *N. branchiphila* were represented within gill-derived amoebae preparations obtained directly from AGD-affected Atlantic salmon, template DNA was amplified using species-specific oligonucleotides (Fig. 2.1A and B, upper). Either *N. pemaquidensis* or *N. branchiphila* were PCR-amplified however amplification was qualitatively variable and PCR product yield was

comparatively lower than products generated from the CCGD strains of either species. Furthermore, PCR products were weakly amplified from only two of the four amoebae primary isolates examined using *N. branchiphila*-specific oligonucleotides (Fig. 2.1A, upper). When *N. pemaquidensis*-specific oligonucleotides were used with template DNA from the same amoebae primary isolates, PCR products were successfully amplified but some variability occurred (Fig. 2.1B upper). Equivalent concentrations of template DNA from cultured strains of *N. pemaquidensis* and *N. branchiphila* consistently amplified PCR products using their respective oligonucleotides with qualitatively more DNA yield than from NCGD amoebae preparations (Fig. 2.1A and B, upper). PCR inhibition may result from differences in culture and isolation techniques (see review Wilson, 1997). All templates were uniformly PCR amplified using universal 18S rRNA gene oligonucleotides, indicating that PCR inhibition did not contribute to the observed variability in PCR amplification (Fig. 2.1A and B, lower).

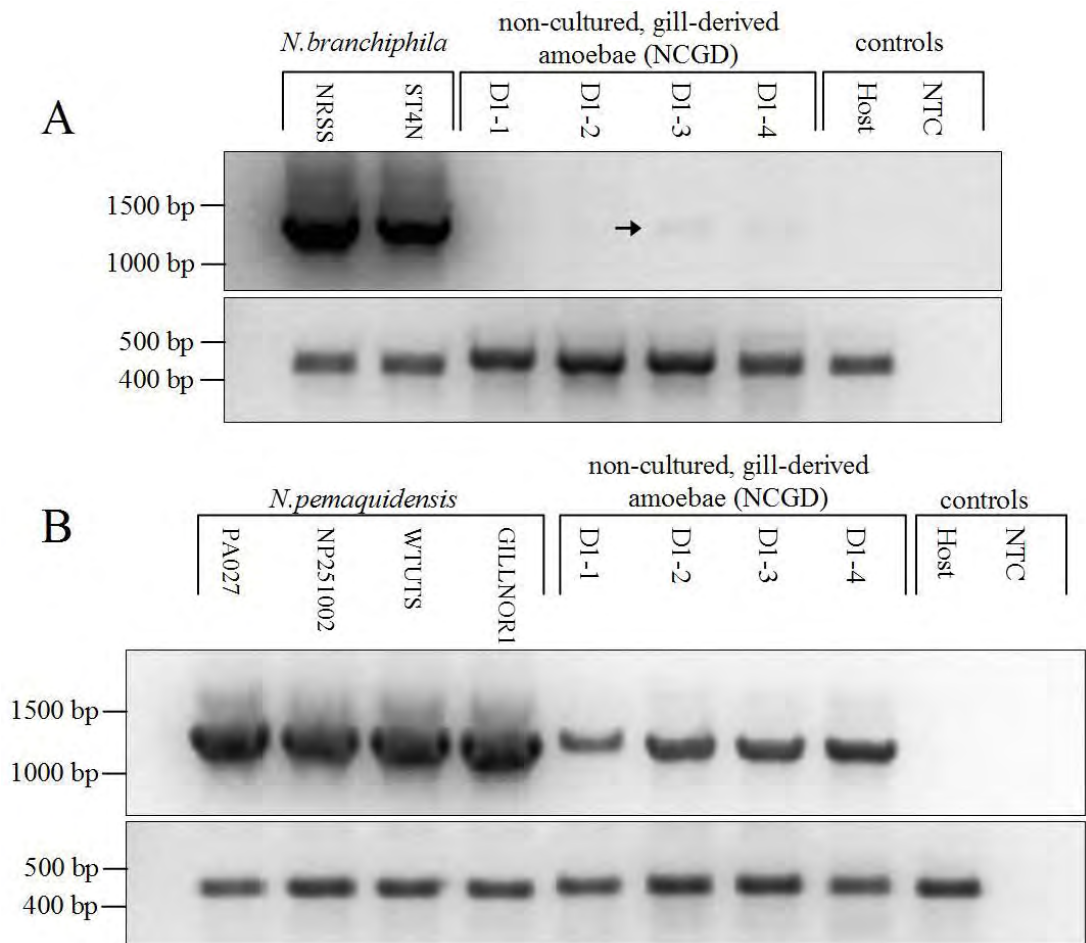


Figure 2.1. Variability in PCR amplification of the 18S rRNA gene from non-cultured, gill-derived (NCGD) amoebae using *Neoparamoeba* species-specific oligonucleotides is not due to PCR inhibition. (A upper) PCR amplification using *N. branchiphila*-specific oligonucleotides. Amoebae DNA templates included *N. branchiphila* strains NRSS and ST4N, four NCGD amoebae primary isolations from AGD-affected Atlantic salmon, *Salmo salar* L. (D1-1 to D1-4), control Atlantic salmon DNA (Host) and no template control (NTC). Arrow shows faint PCR amplicon from NCGD amoebae D1-3 and D1-4. (B upper) PCR amplification using *N. pemaquidensis*-specific oligonucleotides. Amoebae templates included *N. pemaquidensis* strains PA027, NP251002, WTUTS and GILLNOR1, the same NCGD amoebae templates described above (D1-1 to D1-4), control Atlantic salmon DNA (Host) and no template control (NTC). (A and B lower) PCR amplification of identical templates described above using universal 18S rRNA gene oligonucleotides.



#### **2.4.2 Examination of the species-specificity of current *Neoparamoeba* species PCR oligonucleotides**

*Neoparamoeba*-specific oligonucleotides amplify regions of the 18S rRNA gene.

Current *Neoparamoeba* oligonucleotide sequences were compared to their respective annealing sites on the 18S rRNA gene sequences from CCGD and NCGD amoebae as well as Atlantic salmon. PCR amplification and sequencing of the entire 18S rRNA gene of seven molecular clones from five independent primary isolates of NCGD amoebae generated sequences ranging in length from 2044 to 2132 bp with G+C contents between 39.8 and 40.5 %. Consensus 18S rRNA gene sequences were generated for the NCGD amoebae from AGD-affected fish, all described *Neoparamoeba* and Atlantic salmon using mixed base nomenclature standards (NC-IUB, 1985). The consensus sequences were aligned in ClustalX (Thompson et al., 1997) (gap opening/gap extension penalty = 8/2) and the specificity of the *N. pemaquidensis* and *N. branchiphila* oligonucleotide annealing sites were assessed. *N. branchiphila* oligonucleotides aligned specifically with *N. branchiphila* (data not shown) however the *N. pemaquidensis* oligonucleotides partially matched sequences from NCGD amoebae (Fig. 2.2). Of particular note is the infidelity between bases at the 3' end of the sense and antisense oligonucleotides and the template rRNA gene sequences which may partially inhibit PCR amplification.

	<i>N. pemaquidensis</i> sense (5' to 3')	<i>N. pemaquidensis</i> antisense (3' to 5')
	----CATCTCCTTACTAGACTTTCATG----	----CGGGT-AGAGCGAGTTTGTGTG----
<i>N. pemaquidensis</i>	HGTH.....RTTR	CTYTY.....RAGG
<i>N. branchiphila</i>	TATCTT.....WY.....CTATTR	CTCTTTT..G..RRATR.....C....GAGA
<i>N. aestuarina</i>	TRYW.....Y..RATG	YTYT....D-R..RY.....WKKY.GGGA
NCGD amoebae	TGTA.T.....CTATYA	CTYTY....G..A..AT.....R..GAGA
<i>Salmo salar</i>	GCCC.TG.CT..CGGCGCC.CC.---GATG	CTCTT....G..A..AT.....GAGA

Figure 2.2. Alignment of *Neoparamoeba pemaquidensis* 18S rRNA-specific oligonucleotides with known *Neoparamoeba* species and non-cultured, gill-derived (NCGD) amoebae shows 3' infidelity of both the sense and antisense oligonucleotides with the NCGD amoebae 18S rRNA sequence that may have resulted in the variable PCR amplification illustrated in Fig. 2.1. *N. pemaquidensis* forward (fPA -Hxe23a1) and reverse (rPA -Hx49) oligonucleotides described by Wong et al. (2004) aligned against consensus 18S rRNA sequences for known *Neoparamoeba* species, NCGD amoebae isolated from AGD-affected Atlantic salmon, *Salmo salar* and host 18S rRNA. Mixed bases are shown using mixed base nomenclature standards (NC-IUB, 1985). Consensus in bases is represented in the alignment as a dot (.) while a gap in the sequence is represented by a dash (-).

#### 2.4.3 The phylogenetic relationship of NCGD amoebae to *Neoparamoeba* based on 18S rRNA gene sequences

As we observed variability in the 18S rRNA gene sequences from NCGD amoebae along the annealing sites of diagnostic oligonucleotides, we speculated that these sequences may be phylogenetically distinct from other known *Neoparamoeba*. The 18S rRNA gene alignment consisted of 2243 nucleotide sites and of these, 627 were parsimony-informative. The nucleotide similarity values among the 18S rRNA gene sequences are summarised as the range of percent similarity among the phylogenetic lineages examined (Table 2.2). The ML and BI strict consensus trees yielded three distinct lineages within the *Neoparamoeba* genus (clades A, B, & C. Fig. 2.3A).

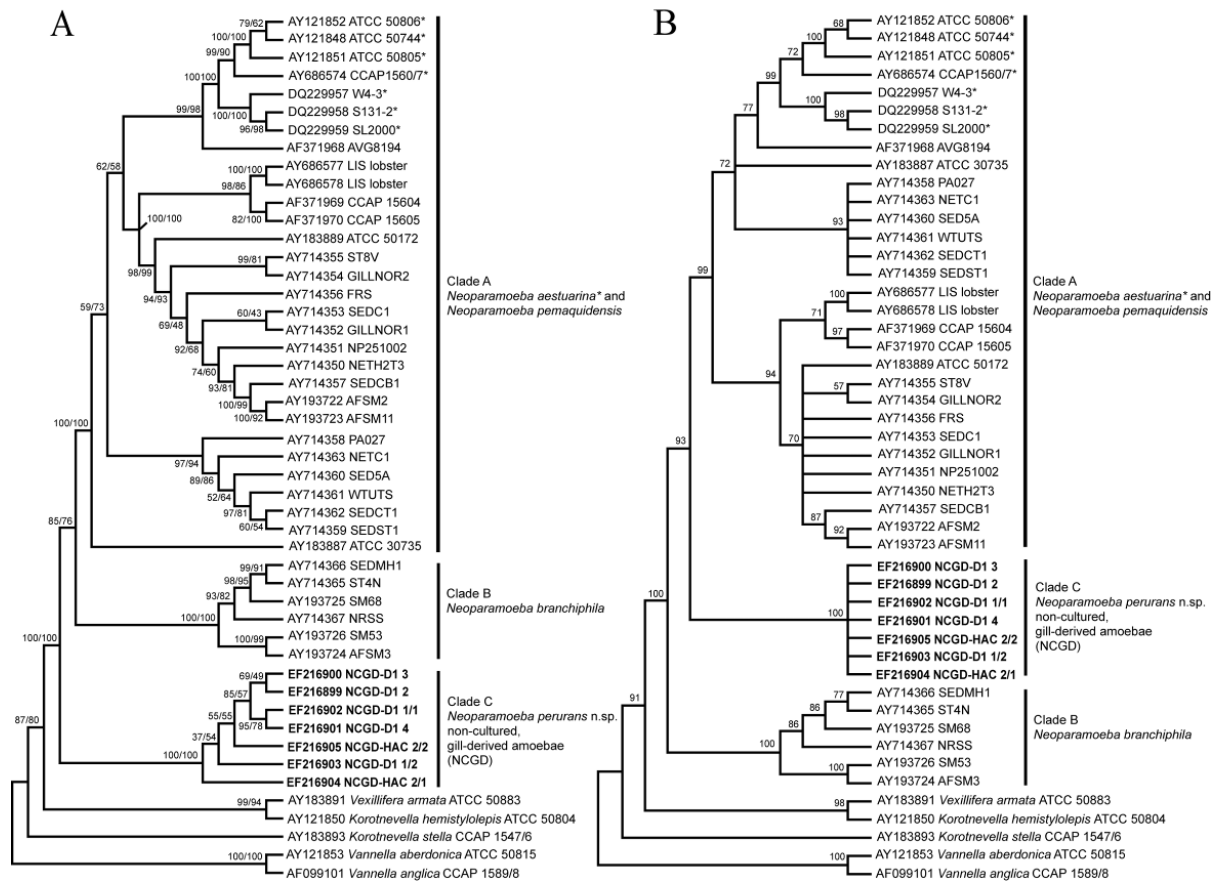


Figure 2.3. Two strict consensus trees resulting from the phylogenetic analysis of *Neoparamoeba* 18S rRNA gene sequences recognise a new genotype of amoeba derived from the gills of AGD-affected Atlantic salmon, *Salmo salar* L. Phylogeny of the *Neoparamoeba* lineage of amoebae was inferred from the 18S rRNA sequences from 41 strains representative of eight taxa and seven molecular clones from five primary isolates from AGD-affected fish (2243 bp of which 627 were parsimony informative) using maximum parsimony (MP), maximum likelihood (ML) and Bayesian phylogenetic inference (BI). BI and ML analyses yielded equally parsimonious consensus trees that distinguished three *Neoparamoeba* clades (Fig. 2.3A). Clade A represents strains of *Neoparamoeba pemaquidensis* and *Neoparamoeba aestuarina* (denoted by an asterix) that grouped into a large, unresolved polyphyletic assemblage. All strains of *Neoparamoeba branchiphila* formed a monophyletic group (Clade B). New 18S rRNA gene sequences (in bold) of non-cultured, gill-derived amoebae (*Neoparamoeba perurans* n. sp.) from AGD-affected Atlantic salmon formed a monophyletic group that was ancestral to all other described *Neoparamoeba* species (Clade C). MP analysis supported the division of *Neoparamoeba* into the same three clades however the strict consensus tree differed in topology with *N. branchiphila* as the ancestral group and differences in the topology within the *N. pemaquidensis*/*N. aestuarina* polyphyletic assemblage (Fig. 2.3B). For each analysis the branch nodes are supported (Fig. 2.3A: BI/ML), ML and MP as percent bootstrap support (1000 replicates) and BI as percentage posterior probability. Both ML and BI approaches implemented a GTR+ $\Gamma$ +I model. BI was run until the standard deviation of split frequencies was <0.01. GenBank accession numbers (<http://www.ncbi.nlm.nih.gov/>) for 18S rRNA gene sequences are shown.

Strains of *N. pemaquidensis* and *N. aestuarina* were unable to be separated and clustered within a large monophyletic group (clade A). There was strong support (76-85%) for *N. branchiphila* strains as a distinct monophyletic group (clade B) while the 18S rRNA gene sequences from NCGD amoebae isolated from tank (NCGD-D1) and field-based (NCGD-HAC) populations of AGD-affected Atlantic salmon were phylogenetically similar. Together, these sequences from NCGD amoebae primary isolates formed a monophyletic group (clade C) that was positioned basal or ancestral to the other described *Neoparamoeba* (100% support). The MP analysis of the 18S rRNA gene sequence alignment yielded a strict consensus tree that was similar to the BI and ML analysis (Fig. 2.3B). Strains of *N. pemaquidensis* and *N. aestuarina* clustered within a similar monophyletic group (clade A) although the clustering among *N. pemaquidensis* strains varied from the tree topology generated by BI and ML analyses. Strains of *N. branchiphila* formed a monophyletic group (clade B, 91% support), positioned as the more divergent of the *Neoparamoeba*. NCGD amoebae isolated from tank (NCGD-D1) and field-based (NCGD-HAC) populations of AGD-affected Atlantic salmon were phylogenetically indistinguishable and formed a well-supported monophyletic group (clade C, 93% support).

#### **2.4.4 Support for a new phylogenetic lineage of *Neoparamoeba* using partial 28S rRNA gene sequence**

To provide additional support for the grouping of phylogenetic lineages inferred from the 18S rRNA genes, partial 28S rRNA gene sequences from representatives of clades A, B, & C described in Fig. 2.3 were PCR-amplified and analysed. A total of

four strains representative of clade A, three strains representative of clade B, six primary isolates representative of clade C and one strain of *P. hoguae* were assessed (Table 2.1). Sequences ranged in length from 1615 to 1686 bp with G+C contents between 38.0 and 41.5%. The 28S rRNA gene alignment consisted of 1750 nucleotide sites and of these, 250 were parsimony-informative. The nucleotide similarity values among the 28S rRNA gene sequences are summarised as the range of percent similarity among the phylogenetic lineages examined (Table 2.2). Phylogenetic analyses of the 28S rRNA gene sequence alignment generated strict consensus trees using BI, MP and ML analyses that converged on identical tree topologies (Fig. 2.4). There was strong support (83-100%) for the exclusion of NCGD amoebae sequences from those representatives of clades A and B in Fig. 2.3, consistent with the preliminary analyses that indicated NCGD amoebae represent a lineage distinct from known *Neoparamoeba*. Representative strains of clade A and B were clustered in well-supported clades (97-100%) as described in Fig. 2.3. There was only modest support (54-61%) for the grouping of clades A and B as sister clades with clade C as the more divergent group, consistent with Fig. 2.3A.

Table 2.2. Percent similarity among the aligned *Neoparamoeba* 18S or 28S rRNA gene sequences used for phylogenetic analyses derived from mean character differences <sup>a</sup>

	<i>N. pemaquidensis</i>	<i>N. aestuarina</i>	<i>N. branchiphila</i>	<i>N. perurans</i>	Outgroup <sup>b</sup>
<i>N. pemaquidensis</i>	96.6 – 100 <b>95.8 - 98.4</b>	93.9 - 96.3	91.1 - 92.6 <b>89.8 - 91.1</b>	92.9 - 94.2 <b>90.8 - 92.3</b>	79.4 - 88.9 <b>73.9 - 74.1</b>
<i>N. aestuarina</i>		95.4 - 99.2	90.31 - 92.0	91.7 - 92.7	79.8 - 88.5
<i>N. branchiphila</i>			96.5 - 98.9 <b>98.1 - 98.3</b>	91.2 - 92.0 <b>89.8 - 90.8</b>	79.5 - 88.6 <b>73.3 - 73.5</b>
<i>N. perurans</i>				98.3 - 99.3 <b>97.3 - 99.6</b>	80.2 - 88.8 <b>74.2 - 74.5</b>
Outgroup <sup>b</sup>					79.5 - 93.3 -

<sup>a</sup> The range of percent sequence similarities are displayed for the 18S and 28S (bold text) rRNA gene sequences among the *Neoparamoeba* species examined

<sup>b</sup> The outgroup for the 18S rRNA gene sequences alignment included *V. armata*, *K. hemistylepis*, *K. stella*, *V. aberdonica*, and *V. anglica*. The outgroup for the 28S rRNA gene sequence alignment was *P. hoguae*

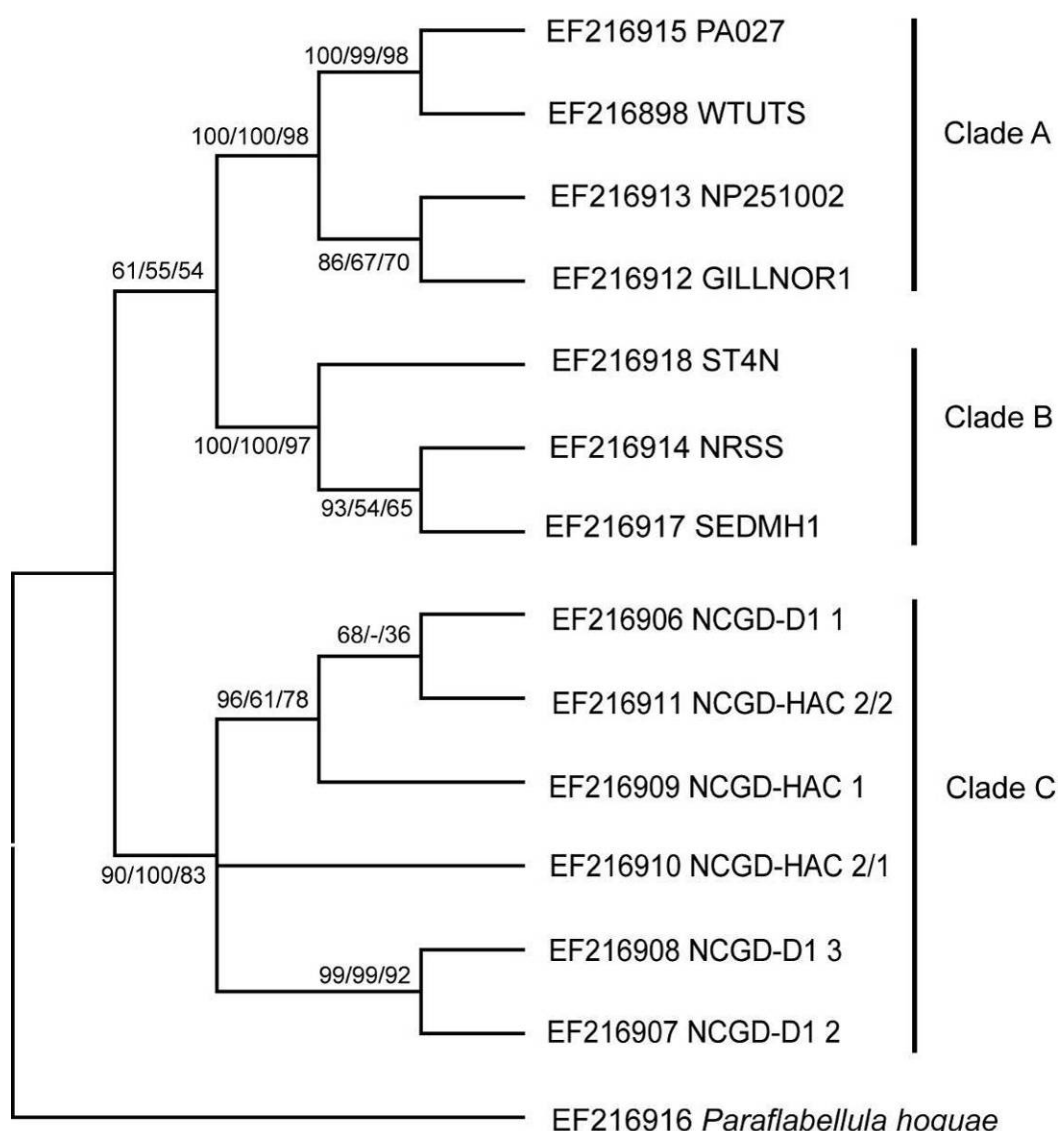


Figure 2.4. Strict consensus tree resulting from the phylogenetic analysis of partial 28S rRNA gene sequences supports the addition of a new genotype to the *Neoparamoeba* genus. Phylogeny of the *Neoparamoeba* lineage of amoebae inferred from 28S rRNA gene sequences (1162 bp of which 250 were parsimony informative) using maximum parsimony (MP), maximum likelihood (ML) and Bayesian phylogenetic inference (BI). Representatives of clades A, B and C described in Fig. 2.3 formed three monophyletic groups and are also designated clades A, B and C in this Figure. Support for each branch is shown (BI/MP/ML), BI as percentage posterior probability and MP and ML as percent bootstrap support (1000 replicates). Both ML and BI approaches implemented a GTR+ $\Gamma$ +I model. BI was run until the standard deviation of split frequencies was <0.01. GenBank accession numbers (<http://www.ncbi.nlm.nih.gov/>) for 28S rRNA gene sequences are shown.

#### **2.4.5 Design and validation of *Neoparamoeba* species-specific oligonucleotide probes for in situ hybridisation**

To identify which phylogenetic lineage(s) of *Neoparamoeba* are associated with AGD, oligonucleotide probes that specifically hybridise rRNA from *N. pemaquidensis*, *N. branchiphila* or the new NCGD amoebae phylogenetic lineage were designed (Fig. 2.5A). To ensure the suitability of fixed tissues for comparative *in situ* hybridisation, a universal 18S rRNA probe (18S probe) was initially hybridised to all tissue sections. The 18S probe hybridised to all amoebae across the *Neoparamoeba* array confirming the integrity of their rRNA (Fig. 2.5B). The specificity of each probe was validated using representative strains from *N. pemaquidensis*, *N. branchiphila* and NCGD amoebae (Fig. 2.5B). Species-specific probes hybridised to their respective representative strain of *Neoparamoeba* with no cross-hybridisation detected. Non-specific signal was not observed on the no-probe control sections (data not shown).



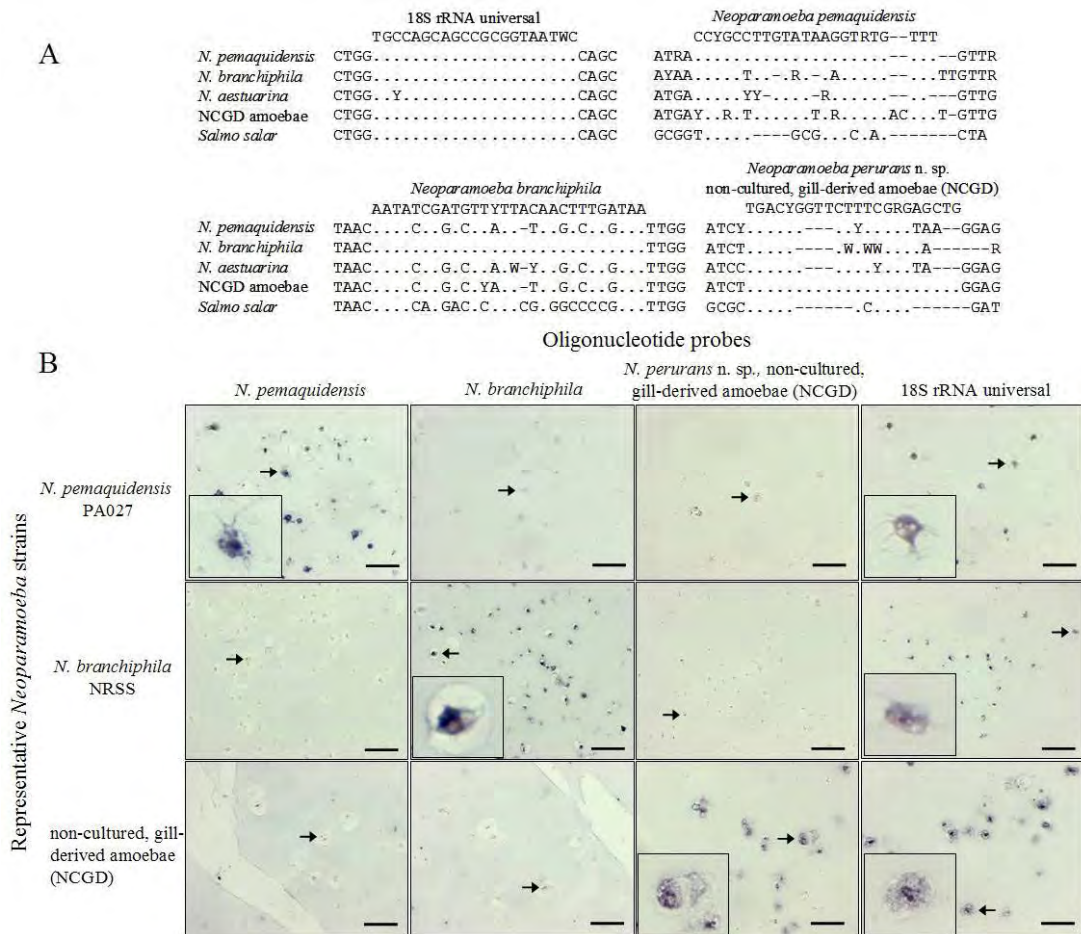


Figure 2.5. Species-specificity of 18S rRNA targeted oligonucleotide probes demonstrated by sequence alignment and *in situ* hybridisation using a *Neoparamoeba* array. (A) Probes aligned to consensus 18S rRNA gene sequences of known *Neoparamoeba* species, non-cultured, gill-derived (NCGD) amoebae isolated from AGD-affected Atlantic salmon, *Salmo salar* L. and Atlantic salmon. Mixed bases are shown using mixed base nomenclature standards (NC-IUB, 1985). Consensus in bases is represented in the alignments as a dot (.) while a gap in the sequence is represented by a dash (-). Probes are presented in the reverse complement orientation to align with the genomic sequences. (B) *In situ* hybridisation showing the genotype-specificity of probes using a *Neoparamoeba* array including representatives from clades A, B and C described in Fig. 2.3 and 2.4. *N. pemaquidensis* (PA027), *N. branchiphila* (NRSS) and NCGD amoebae from AGD-affected Atlantic salmon are shown. Universal 18S rRNA probes hybridised to all *Neoparamoeba* in the array and genotype-specific probes only hybridised to their targeted genotype. Arrows highlight amoebae and probe-positive cells are magnified within the inserts. Immunological detection using DIG-labelled oligonucleotide probes and BCIP/NBT, counterstained using 0.1% fast red. Scale bars represent 50  $\mu$ m. Non-specific signal was not observed on the no-probe control sections (data not shown).

#### **2.4.6 Description of AGD aetiology by hybridising species-specific oligonucleotide probes to amoebae associated with AGD-lesions**

The role of the three phylogenetic lineages of *Neoparamoeba* described in this study (clades A, B and C, Fig. 2.3) in AGD aetiology was assessed by hybridising species-specific probes to amoebae associated with typical AGD lesions. All serially sectioned gill arches from three tank and four field-based Atlantic salmon presented with typical AGD lesions as previously described (reviewed by Munday et al., 2001). Sections of gill filaments presented with hyperplasia of epithelia-like cells resulting in lamellar fusion. Amoebae with at least one intracellular PLOs were adjacent to these lesions (Fig. 2.6). The universal 18S probe hybridised evenly to rRNA in gill tissues and amoebae trophozoites, confirming the rRNA integrity in tissues across all sections (data not shown). In all tank and field-based AGD-affected Atlantic salmon gill tissues, the NCGD amoebae-specific probe hybridised with amoebae associated with typical AGD-lesions and unambiguously associated the new phylogenetic lineage (clade C) with AGD (Fig. 2.6). The *N. pemaquidensis* and *N. branchiphila* probes did not hybridise with sectioned amoebae on the gill arches and non-specific signal was not observed on the no-probe control sections (data not shown).

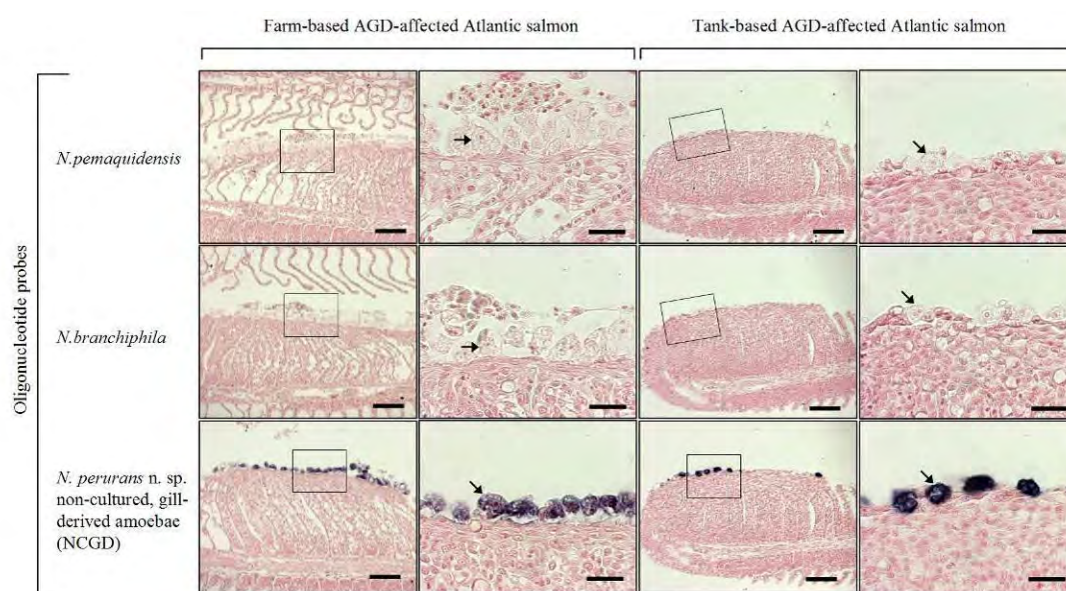


Figure 2.6. A fourth genotype of *Neoparamoeba* is associated with gill lesions from AGD-affected Atlantic salmon, *Salmo salar* L.. Genotype-specific oligonucleotide probes that hybridise to 18S rRNA of *Neoparamoeba pemaquidensis*, *Neoparamoeba branchiphila* and non-cultured, gill-derived (NCGD) amoebae (*N. perurans* n. sp.) were used to probe gill tissue from tank and field-based AGD-affected Atlantic salmon. Images are representative of *in situ* hybridisation experiments using a total of three tank-based and four farm-based AGD-affected fish (i.e. n = 7 fish). Serial sections of gill filaments with typical AGD lesions with amoebae attached to the lesion surface (arrows). Probes that hybridised to amoebae are blue. Inset boxes are magnified in the adjacent image. Immunological detection using DIG-labelled oligonucleotide probes and BCIP/NBT, counterstained using 0.1% fast red. Low magnification scale bar represents 100  $\mu$ m, high magnification represents 30  $\mu$ m. Non-specific signal was not observed on the no-probe control sections (data not shown).

## 2.4.7 Description of *Neoparamoeba perurans* n. sp. (Lobosea; Vexilliferidae)

### 2.4.7.1 Description

*N. perurans* n. sp. morphology corresponds to previous descriptions of *Neoparamoeba* species (Dyková et al., 2005b; Page, 1987). Amoebae with digitiform pseudopodia when free floating, and mamilliform pseudopodia when adhered. Trophozoites 41-56  $\mu$ m in adhered form. One or more *Perkinsiella amoebae*-like organisms (5.3-8.0  $\mu$ m) adjacent to nucleus (3.3-6.0  $\mu$ m) and cell-surface microscales

absent. Histologically, visible nucleus and parasome present in trophozoites associated with host gill tissue. Cytoplasm vacuolated. Member of an exclusive phylogenetic cluster within the *Neoparamoeba* genus based on 18S and 28S rRNA gene sequenced from this species (GenBank accession numbers EF216898-EF216918).

#### **2.4.7.2 Host**

Type host Atlantic salmon, *Salmo salar* L. 1758. (Salmoniformes: Salmonidae). Trophozoites attached to gills adjacent to hyperplastic epithelia-like cells. Atlantic salmon reared in recirculating tank system infected with non-cultured, gill-derived amoebae by cohabitation with AGD-affected Atlantic salmon. Atlantic salmon reared in sea-cages obtained during AGD epizootic. In both cases, *N. perurans* n. sp. was the only amoeba identified in association with gill lesions.

#### **2.4.7.3 Locality**

Confirmed cases of *N. perurans* n. sp. infections are from the D'Entrecasteaux Channel, Tasmania, Australia.

#### **2.4.7.4 Type material**

Type material consisting of frozen and fixed *N. perurans* n. sp. derived from host gill tissue are held in the collection of the University of Tasmania, Australia. H&E stained histological sections of Atlantic salmon gill tissue, confirmed by *in situ* hybridisation to be infected with *N. perurans* n. sp., were deposited in the collection of the Queensland Museum, Brisbane, Australia. Eighteen syntypes were deposited

as six serial tissue sections from each of three fish (Fish 1: G464935-G464940, Fish 2: G464941-G464946, Fish 3: G464947-G464952). The 18S and 28S rRNA gene sequences of gill-derived amoebae were deposited in GenBank (accession numbers EF216898-EF216918).

#### **2.4.7.5 Etymology**

This species is named after the Latin word for inflame, representing the inflammation associated with attachment of amoebae to gill lamellae.

### **2.5 Discussion**

In earlier studies, numerous strains of *N. pemaquidensis* and *N. branchiphila* were successfully cultured from AGD-affected fish (Dyková et al., 2005b; Dyková et al., 2007; Fiala and Dyková, 2003). The morphological similarities of these cultured strains with trophozoites associated with AGD gill lesions led to the belief that both species could be causal in AGD (Dyková et al., 2005b; Wong et al., 2004) however the parasite-disease nexus has never been directly corroborated. In this study, our initial objective was to address the aetiological role of *N. pemaquidensis* and *N. branchiphila* in AGD. Through a molecular approach, a new phylogenetically distinct lineage of *Neoparamoeba* was identified and shown to be the exclusive aetiological agent of AGD in all samples examined. Therefore, we describe this new lineage as *Neoparamoeba perurans* n. sp. on the basis of both phylogenetic and virulence-related phenotypic divergence from other *Neoparamoeba* species.

Amoebae first assumed to be *N. pemaquidensis* and *N. branchiphila* were found in preparations of amoebae directly isolated from AGD-affected Atlantic salmon by PCR. However, amplification of NCGD amoebae DNA by PCR using *N. pemaquidensis* and *N. branchiphila*-specific oligonucleotides yielded comparatively lower amplicons, suggesting these were not the predominant species. This observation prompted the sequencing of the 18S rRNA gene from NCGD amoebae and the subsequent recognition that there was variation in NCGD amoebae sequences initially within the *N. pemaquidensis* and *N. branchiphila* oligonucleotide annealing sites. Thus the PCR results for *N. pemaquidensis* may have been confounded by the possibility that the *N. pemaquidensis* oligonucleotides partially amplify more than one species of *Neoparamoeba*. While species-specific PCR amplification may provide a rapid diagnostic tool to confirm the presence of *Neoparamoeba* in DNA preparations (Dyková et al., 2005b; Wong et al., 2004), it is strictly dependent on oligonucleotide specificity. Until the *N. pemaquidensis* oligonucleotides are validated, particularly with respect to the amplification of the NCGD amoebae rRNA gene, their utility is questionable and further use without redesign is cautioned.

Qualitative assessment of *N. pemaquidensis* and *N. branchiphila* in amoebae preparations from AGD-affected fish using PCR led to the sequencing of 18S rRNA genes from NCGD amoebae isolates. These sequences were used to phylogenetically assess NCGD amoebae and closely-related taxa. NCGD amoebae sequences clustered together with sequences from amoebae of the *Neoparamoeba* genus, conforming with the taxonomic classification of NCGD amoebae assigned using morphological characters (Morrison et al., 2004; Morrison et al., 2005). However no

further morphometric evaluation of NCGD amoebae was performed given that sub-generic discrimination of *Neoparamoeba* using morphological features is unreliable (Dyková et al., 2005b). Phylogenetic analyses of the 18S rRNA gene generated a monophyletic group exclusive to NCGD amoebae sequences. In addition, analyses of the 28S rRNA gene from representatives of the three phylogenetically distinguishable lineages were consistent with this preliminary inferred taxonomic classification where NCGD were excluded from other *Neoparamoeba* sequences.

During phylogenetic analyses, *N. pemaquidensis* and *N. aestuarina* sequences were not resolved into monophyletic groups as previously described (Dyková et al., 2005b; Fiala and Dyková, 2003). The segregation of *N. pemaquidensis* and *N. aestuarina* based on a comparison of their 18S rRNA genes has already proven difficult. Following the inclusion of additional 18S rRNA gene sequences of *Neoparamoeba* and related taxa, the phylogenetic relationship between the *N. pemaquidensis* strain AVG 8194 and the *N. aestuarina* group became ambiguous (Dyková et al., 2007). On a broader scale, when *Neoparamoeba* 18S rRNA gene sequences were compared with similar sequences from a wide selection of protist taxa there was diminishing support for the separation of *N. pemaquidensis* and *N. aestuarina* (Fiala and Dyková, 2003; Peglar et al., 2003). It was suggested that the inability to distinguish *N. pemaquidensis* from *N. aestuarina* was due to the elimination of phylogenetically informative sites from the analyses after they could not be aligned with the non-*Neoparamoeba* species (Peglar et al., 2003). Here, the inability to distinguish previously described lineages of *Neoparamoeba* using the 18S rRNA gene is possibly an artefact of poor resolution among closely-related

species. While the highly conserved nature of 18S rRNA genes across the animal kingdom allows the comparison of divergent taxa (Hillis and Dixon, 1991), it also limits the power to discriminate between closely-related species (Adam et al., 2000). For example, the relationship between strains of the *Acanthamoeba* genus remained unresolved using comparisons of the 18S rRNA gene (Stothard et al., 1998). Alternative regions of the rRNA gene with higher rates of variability between closely related taxa such as the 28S and internal transcribed spacer 1 (ITS1) regions have been used to distinguish inter- and intra-specific relations (for example Adam et al., 2000; Bergholtz et al., 2005; Hansen et al., 2000; Hosoi-Tanabe et al., 2006; Köhler et al., 2006; Sato et al., 2005). In the present study, partial 28S rRNA gene sequences spanning the phylogenetically informative D1 to D3 domains were compared. Sequencing the partial 28S rRNA gene from additional cultured *Neoparamoeba* strains, NCGD amoebae primary isolates and strains of closely related taxa may be more useful to resolve the phylogeny of the entire *Neoparamoeba* lineage.

Given that there now was indirect evidence that up to three species of *Neoparamoeba* were associated with AGD-affected fish, we sought to resolve which of these species were responsible for AGD. Previously, it was suggested that an ISH-based test for histological sections would be the most suitable tool for prospective as well as retrospective AGD aetiological studies (Dyková et al., 2005b). Therefore during our study, we adopted this approach whilst fulfilling the sequence-based guidelines for microbial disease causation (Fredricks and Relman, 1996). Using ISH, *N. perurans* n. sp. but not *N. pemaquidensis* and *N. branchiphila* was unambiguously identified as the predominant pathogenic amoeba adjacent to the gill-lesions of AGD-affected



Atlantic salmon in Tasmania. As neither *N. pemaquidensis* nor *N. branchiphila* have been demonstrably associated with AGD gill lesions, or shown to induce AGD (Howard and Carson, 1993b; Kent et al., 1988; Morrison et al., 2005; Vincent et al., 2007) there remains a contrasting representation of *Neoparamoeba* species from *in vitro* and *in vivo* studies. Whether there is selection of *Neoparamoeba* species during continuous *in vitro* culture is unknown but warrants further investigation.

Since the representation of *Neoparamoeba* species differs between *in vitro* culture and *in vivo* infection, it was pertinent to investigate whether the species composition could alter when virulent *Neoparamoeba* were maintained using cohabitation of fish in a recirculation tank for approximately five years. Data presented here clearly show congruent 18S and 28S rRNA gene sequences of NCGD amoebae isolated from tank and field-based populations of Atlantic salmon. In addition, *N. perurans* n. sp. was the only detectable species associated with AGD lesions in samples obtained from both populations of fish. This indicates that experimentally-induced AGD in tank-based infectivity trials reflect the AGD aetiology observed in field-based populations of AGD-affected Atlantic salmon in Tasmania and that *N. perurans* n. sp. may have been the predominant species of amoeba responsible for the experimental induction of AGD in previously published studies (for example Adams and Nowak, 2004a; Bridle et al., 2003; Bridle et al., 2006a; Embar-Gopinath et al., 2005; Gross et al., 2004b; Gross et al., 2005; Morrison et al., 2006a; Morrison et al., 2004; Morrison et al., 2005; Vincent et al., 2006; Zilberg and Munday, 2001).

In summary, a molecular-based approach led to the discovery of *N. perurans* n. sp. that was linked to cellular pathology in AGD-affected Atlantic salmon. This provides compelling evidence of causation, consistent with the sequence-based identification of microbial pathogens guidelines (Fredricks and Relman, 1996). The spatial and temporal distribution of *Neoparamoeba* in the context of AGD is unknown; however data presented here highlight the need to incorporate culture-independent methods in future studies.

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### **3    *NEOPARAMOEBA PERURANS* IS A COSMOPOLITAN AETIOLOGICAL AGENT OF AMOEBIC GILL DISEASE**

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*Neoparamoeba perurans* is a cosmopolitan aetiological agent of amoebic gill  
disease. *Diseases of Aquatic Organisms* **78**, 217-223.

### 3.1 Abstract

Previously, we described a new member of the *Neoparamoeba* genus, *N. perurans* and showed that it is an agent of amoebic gill disease (AGD) of Atlantic salmon cultured in South-East Tasmania, Australia. Given the broad distribution of cases of AGD, we were interested in extending our studies to epizootics in farmed fish from other sites around the world. Oligonucleotide probes that hybridise with the 18S rRNA of *N. perurans*, *N. branchiphila* or *N. pemaquidensis* were used to examine archival samples of AGD in Tasmania as well as samples obtained from four host fish species cultured across six countries. In archival samples, *N. perurans* was the only detectable amoeba, confirming that it has been the predominant aetiological agent of AGD in Tasmania since epizootics were first reported. *N. perurans* was also the exclusive agent of AGD in four host species across six countries. Together, these data show that *N. perurans* is a cosmopolitan agent of AGD and therefore of significance to the global mariculture industry.

### 3.2 Introduction

Amoebic gill disease (AGD) is an ectoparasitic condition of some farm-reared marine fish (Dyková et al., 1995; Kent et al., 1988; Munday, 1986). The first AGD epizootic was reported by Munday (1986) and affected both rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) cultured in South-East Tasmania, Australia. At that time the pathogen was not identified, but later, using morphological evidence, the aetiological agent was described as *Paramoeba* sp. (Roubal et al., 1989). In the interim, Kent et al. (1988) described *P. pemaquidensis* (now *Neoparamoeba pemaquidensis*) as the agent of AGD of Coho salmon (*Oncorhynchus kisutch*) farmed in Washington State, USA.

Many authors assumed that *N. pemaquidensis* was the sole aetiological agent of AGD of Atlantic salmon (Adams and Nowak, 2001; Bowman and Nowak, 2004; Clark et al., 2003; Douglas-Helders et al., 2001a; Douglas-Helders et al., 2002; Douglas-Helders et al., 2003a; Douglas-Helders et al., 2005; Douglas-Helders et al., 2000; Douglas-Helders et al., 2003b; Douglas-Helders et al., 2001b; Douglas-Helders et al., 2003c; Munday et al., 2001; Powell and Clark, 2003; Powell and Clark, 2004; Tan et al., 2002; Wong et al., 2004; Zilberg, 2005). However, the isolation of *N. branchiphila* from the gills of AGD-affected fish (Dyková et al., 2005b) suggested that AGD may be a condition of mixed aetiology. While *N. pemaquidensis* and *N. branchiphila* were the predominant species of amoebae cultured from the gills of AGD-affected fish, neither have been shown to induce AGD in fish that were experimentally inoculated with clonal cultured strains (Howard and Carson, 1993a; Kent et al., 1988; Morrison et al., 2005; Vincent et al.,

2007). Recently, *N. perurans* was discovered and shown to be an agent of AGD in Atlantic salmon cultured in South-East Tasmania (Young et al., 2007). In this instance, neither *N. pemaquidensis* nor *N. branchiphila* were detected in sections of gill tissue affected by AGD. In cases of AGD reported elsewhere, it is not known what role *N. perurans*, *N. pemaquidensis* and/or *N. branchiphila* play. Therefore in this study, our objective was to use species-specific molecular probes to determine the aetiological agent(s) of AGD in four host species across six countries.

### **3.3 Materials and Methods**

Paraffin-embedded gill tissues were obtained from four fish species predominantly during or following epizootics at commercial fish-farming operations across six countries (Table 3.1). An epizootic was not reported from Chinook salmon (*Oncorhynchus tshawytscha*) cultured in New Zealand. However, the smallest fish (runts) within the healthy population were observed to have gill lesions that corresponded with AGD and these fish were used in this study. Gill tissues were sectioned (3-7 µm), stained with haematoxylin and eosin and examined using light microscopy. Alternatively, sections (7 µm) of gill tissues were placed onto Polysine glass slides (Menzel-Gläser, Braunschweig, Germany) and dried overnight at 37°C. Sections were hybridised with a digoxigenin (DIG)-labelled “universal” 18S rRNA oligonucleotide probe to verify the integrity of rRNA as previously described (Young et al., 2007). All gill tissues with suitable host and amoeba rRNA were serially-sectioned, placed onto Polysine glass slides and incubated with *N. perurans*, *N. branchiphila* and *N. pemaquidensis* DIG-labelled oligonucleotide probes as previously described (Young et al., 2007). Positive and negative (no probe) controls

were run in parallel with each *in situ* hybridisation experiment by hybridising each probe with a section containing representative strains of each *Neoparamoeba* species termed an “amoebae array” as previously described (Young et al., 2007). Tissue sections were incubated for up to 1 h with premixed BCIP/NBT solution (Sigma-Aldrich, Castle Hill, New South Wales, Australia) for colour development. The hybridisation procedure for the gill tissue sections from AGD-affected turbot, *Psetta maxima* was modified as gill tissue was provided pre-sectioned (5 µm) on non-coated slides. Preliminary hybridisation experiments with these sections resulted in tissues detaching from the slides. Therefore the prehybridisation procedure was limited to dewaxing, rehydration and sequential washes of sections for 2× 5 min with diethyl pyrocarbonate -treated PBS at room temperature. Sections were then directly probed with *N. perurans*, *N. branchiphila* and *N. pemaquidensis* oligonucleotides as previously described (Young et al., 2007). Positive and no probe controls were included with each *in situ* hybridisation experiment as described above. An extended incubation (18 h) with premixed BCIP/NBT solution (Sigma-Aldrich) was required for colour development.

Table 3.1. Gill tissue samples obtained from farm-reared populations of fish with presumptive cases of amoebic gill disease. The species, location and year gill tissues were originally sampled are provided.

Species	Location <sup>a</sup>	Year	n <sup>b</sup>	Fixative <sup>c</sup>
Atlantic salmon, <i>Salmo salar</i>	Bruny Island, South-East Tasmania, Australia	1987	1	SWD
	Brabazon Point, South-East Tasmania, Australia	1987	1	SWD
	Tamar River, North Tasmania, Australia	2006	3	NBF
		2007	1	SWD
	Galway, Ireland	2004	3	NBF
	Puget Sound, Washington State, U.S.A	2006	3	NBF
	North Uist, Western Isles, Scotland	2006	4	SWF
Rainbow trout, <i>Oncorhynchus mykiss</i>	Recherche Bay, South-East Tasmania, Australia	1988	1	SWD
Chinook salmon, <i>Oncorhynchus tshawytscha</i>	Picton, Queen Charlotte Sound, New Zealand	2006	3	NBF
Turbot, <i>Psetta maxima</i>	North-West Spain	2001	1	SWD

<sup>a</sup> The geographical location of samples obtained from Australia is specified to denote the division between North and South Tasmania.

<sup>b</sup> Number of fish sampled at each time point.

<sup>c</sup> Fixative used to prepare gill tissues for histology: seawater Davidson's fixative (SWD), 10% formalin prepared in seawater (SWF) or 10% neutral buffered formalin (NBF).

### 3.4 Results and Discussion

When Dyková et al. (2000) described several strains of *Neoparamoeba* from AGD-affected European bass, *Dicentrarchus labrax* and turbot, the authors suggested that the agents of AGD should only be assigned to *Neoparamoeba*, since members of the



genus (*N. pemaquidensis* and *N. aestuarina*) were morphologically identical. Since then, another morphologically indistinct species from the genus *Neoparamoeba* (*N. branchiphila*) was cultured from AGD-affected fish (Dyková et al., 2005b) and therefore at this point, up to three *Neoparamoeba* species were potential aetiological agents of AGD. Recently, we described a new species *N. perurans* and showed that it is an agent of AGD of Atlantic salmon from South-East Tasmania (Young et al., 2007). This resolved AGD aetiology in recent cases of AGD in Atlantic salmon cultured in South-East Tasmania but not in historical cases of AGD from South-East Tasmania, nor in cases described elsewhere.

In this study, cases of AGD were verified by histological examination (Dyková and Novoa, 2001). All histological sections revealed structural changes that were consistent with the pathology described in cases of AGD (Dyková et al., 1995; Kent et al., 1988; Munday et al., 1990; Rodger and McArdle, 1996; Roubal et al., 1989). The most significant change was the development of gill lesions due to the hyperplasia of epithelial-like cells (Figs. 3.1, 3.2 and 3.3). This resulted in extensive secondary lamellar fusion and the formation of interlamellar vesicles (Figs. 3.2 and 3.3). Amoebae were located between the secondary lamellae or at the distal ends of fused lamellae (Figs. 3.1, 3.2 and 3.3). The presence of a perinuclear eosinophilic body was representative of the endosymbiotic *Perkinsiella amoebae*-like organism (PLO) (Dyková et al., 2003) (Fig. 3.1 a-i) and was identified in amoebae in all sections examined.

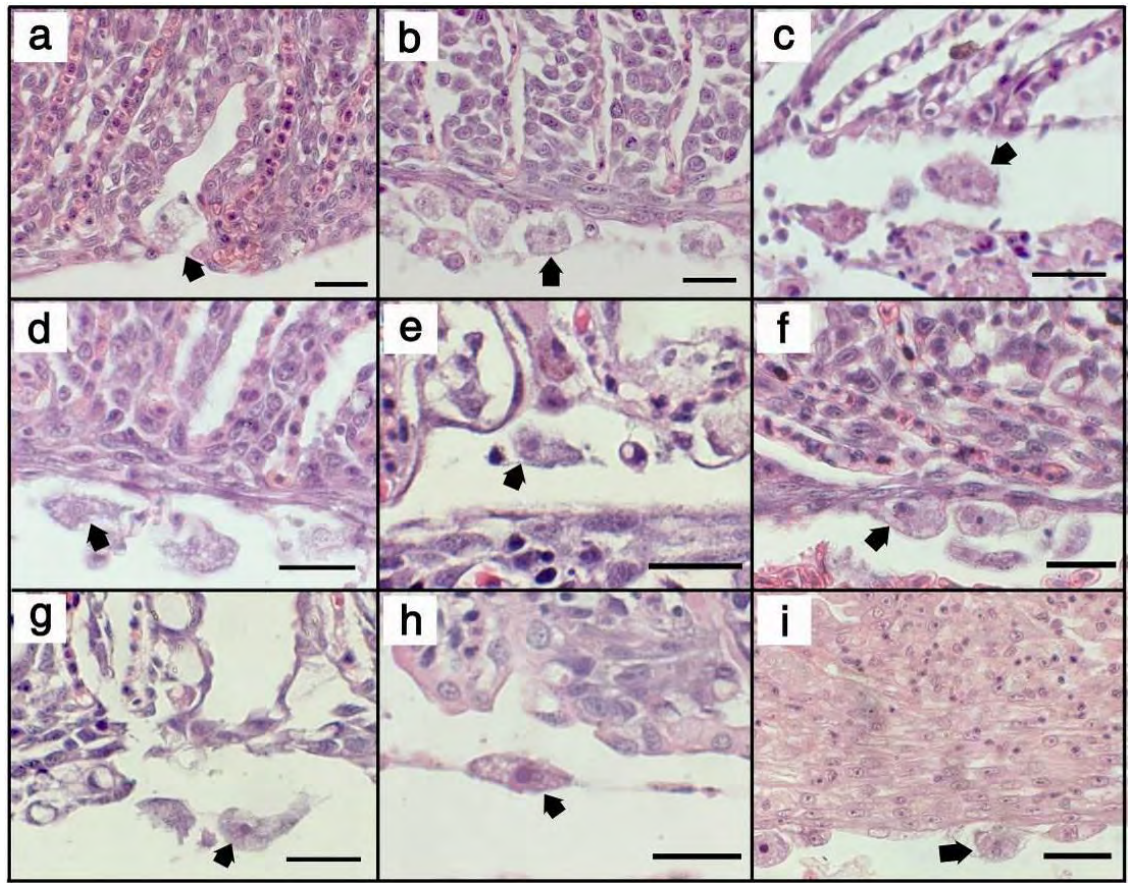


Figure 3.1. Confirmation of the presence of amoebae (arrows) containing endosymbiotic *Perkinsiella amoebae*-like organisms in H&E stained, 3–7  $\mu\text{m}$  thick sections from fish examined in this study. Images are representative of the pathology observed in specimens from each species and location. (a) *Salmo salar*, Tamar River, North Tasmania, Australia (n=4). (b) *S. salar*, Bruny Island, South-East Tasmania, Australia, 1987 (n=1). (c) *S. salar*, Brabazon Point, South-East Tasmania, Australia, 1987 (n = 1). (d) *Oncorhynchus mykiss*, Recherche Bay, South-East Tasmania, Australia, 1988 (n = 1). (e) *S. salar*, Galway, Ireland (n=3). (f) *S. salar*, Western Isles, Scotland (n = 4). (g) *S. salar*, Washington, U.S.A (n=3). (h) *O. tshawytscha*, Picton, New Zealand (n = 3). (i) *Psetta maxima*, North-West Spain (n=1). Scale bars on each image represent 25  $\mu\text{m}$ .

Retrospective studies applying *in situ* hybridisation probes to tissue samples have been used to determine the aetiological agents of disease and the geographical and temporal distribution of marine diseases (Friedman et al., 2005; Hasson et al., 1999; Yee et al., 2005). Here, archival tissues from cultured Atlantic salmon and rainbow trout in South-East Tasmania, Australia were probed for *N. perurans*, *N.*

*branchiphila* and *N. pemaquidensis*. These samples were obtained in 1987 and 1988, coinciding with the first confirmed records of AGD epizootics (Munday, 1986; Roubal et al., 1989). In South-East Tasmania, recurrent AGD epizootics occur (Munday et al., 1990; Munday et al., 1993; Roubal et al., 1989) and fish are repeatedly treated sometimes more often than monthly during summer. Therefore we sought to clarify if there was temporal change in *Neoparamoeba* species associated with AGD in South-East Tasmania. The *N. perurans*-specific probe hybridised with all trophozoites in all sections examined (Fig. 3.2 A-C) consistent with samples obtained in recent cases of AGD in this region (Young et al., 2007). In serially-sectioned gill tissues, neither the *N. pemaquidensis* nor the *N. branchiphila*-specific probes hybridised with any trophozoites (Fig. 3.2 A-C). *Neoparamoeba* species-specific probes hybridised with the corresponding *Neoparamoeba* species on the amoebae array while no signal was detected in trophozoites on the amoebae array when the probes were omitted from the hybridisation procedure. This occurred in all hybridisation experiments. In northern Tasmania, there is a single Atlantic salmon farm, located on the Tamar River. During the 2006/7 summer, an AGD epizootic occurred and in all samples obtained from affected fish, *N. perurans* was the only detectable amoeba (Fig. 3.2 D). Together, these data suggest that *N. perurans* has been and remains the predominant aetiological agent of AGD throughout Tasmania.

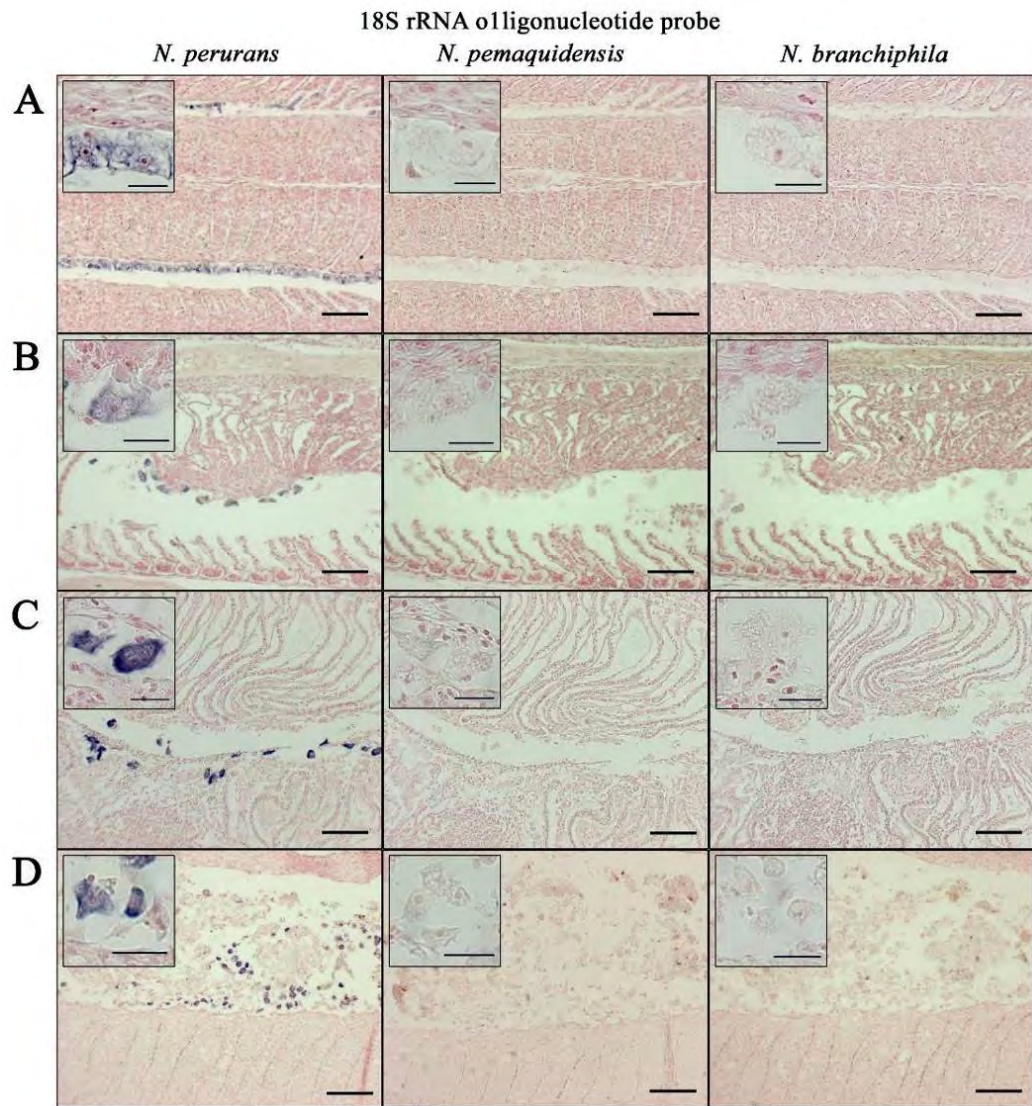


Figure 3.2. Results of *in situ* hybridisation experiments based on species-specific probes that hybridise to the 18S rRNA of either *Neoparamoeba perurans*, *N. pemaquidensis* or *N. branchiphila* in gill samples from fish cultured in Tasmania, Australia. Images are representative of the pathology observed in specimens from each species and location. (A) *Salmo salar*, Bruny Island, South-East Tasmania, 1987 (n=1). (B) *S. salar*, Brabazon Point, South-East Tasmania, 1987 (n=1). (C) *Oncorhynchus mykiss*, Recherche Bay, South-East Tasmania, 1988 (n=1). (D) *S. salar*, Tamar River, North Tasmania, 2006 (n=4). Probe-positive and probe-negative amoebae are magnified within the insets. Scale bars on each image represent 100  $\mu\text{m}$  or 20  $\mu\text{m}$  (insets).



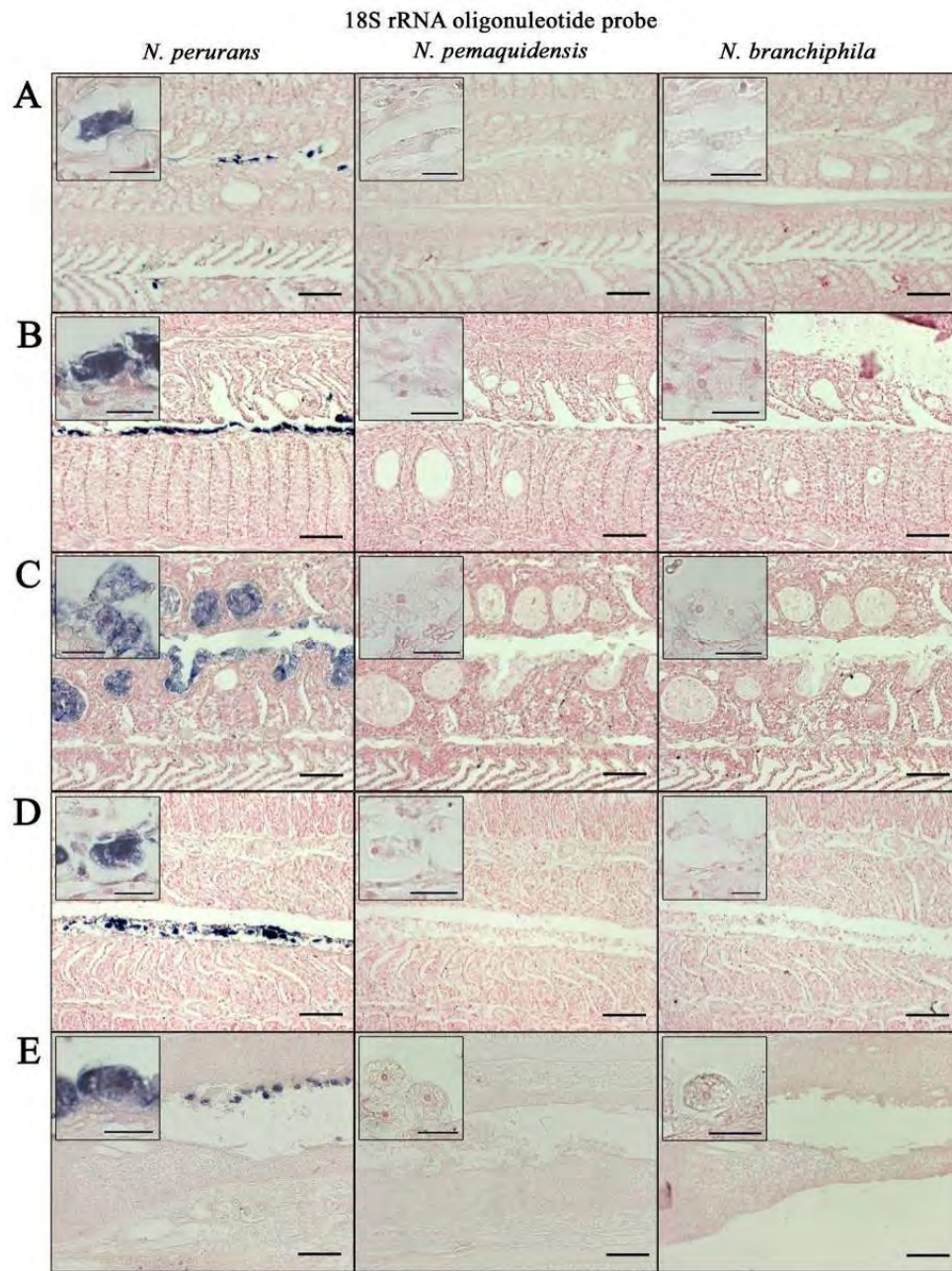


Figure 3.3. Results of *in situ* hybridisation experiments based on species-specific probes that hybridise to the 18S rRNA of either *Neoparamoeba perurans*, *N. pemaquidensis* or *N. branchiphila* in gill samples from fish sampled world-wide. Images are representative of the pathology observed in specimens from each species and location. (A) *Oncorhynchus tshawytscha*, Picton, New Zealand (n=3). (B) *Salmo salar*, Galway, Ireland (n=3). (C) *S. salar*, Western Isles, Scotland (n=4). (D) *S. salar*, Washington State (n=3). (E) *Psetta maxima*, North-West Spain (n=1). Images are representative of *in situ* hybridisation experiments using one to four AGD-affected fish from each location (see Table 2.1). Probe-positive and probe-negative amoebae are magnified within the insets. Scale bars on each image represent 100  $\mu$ m or 20  $\mu$ m (insets).

There have been numerous published and unpublished reports of AGD in cultured fish (Nowak et al., 2002). Several hosts are susceptible and there is a world-wide distribution of cases (see review by Munday et al., 2001). Many salmonids are susceptible to AGD and here, cases of AGD in Atlantic salmon, rainbow trout and Chinook salmon were verified and the sole agent confirmed as *N. perurans* (Figs. 2 and 3). The locations of these cases were Tasmania (Atlantic salmon and rainbow trout), Galway (Atlantic salmon) and Washington State (Atlantic salmon). In addition, the first formal case of AGD in Atlantic salmon from Scotland is presented, together with a case of AGD in runt Chinook salmon from New Zealand, confirming the observations of AGD-like gill lesions in fish from this location (Howard and Carson, 1993b; Munday et al., 1990). Other than salmonids, AGD affects turbot (Dyková et al., 1995; Dyková et al., 1998) and the agent in one case from North-West Spain is confirmed here as *N. perurans*. Some background signal was detected in sections of turbot gill tissue. This was due to the extended colour development (18 h) which resulted in a non-specific pale blue colouration of cartilaginous tissues. These tissues were not associated with primary or secondary gill lamellae or trophozoites (data not shown).

In summary, we used a molecular-based procedure to probe histological sections obtained from four host fish species cultured across six countries. In all 21 specimens, *N. perurans* was the only species of *Neoparamoeba* shown to elicit AGD, confirming that this species is a cosmopolitan protozoan parasite of the temperate marine fish examined. Confirmation of *N. perurans* in regions of significant finfish production indicates that AGD is of global significance to the mariculture industry.

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## **4 COEVOLUTION OF *NEOPARAMOEBA* AND THEIR ENDOSYMBIONTS, *PERKINSELA AMOEBAE*-LIKE ORGANISMS**

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Coevolution of *Neoparamoeba* and their endosymbionts, *Perkinsela amoebae*-like  
organisms.

#### 4.1 Abstract

Neoparamoeba are defined by an obligate symbiosis with their endosymbionts, *Perkinsela amoebae*-like organisms (PLOs). Phylogenetic congruence between *Neoparamoeba pemaquidensis*, *Neoparamoeba branchiphila* and *Neoparamoeba aestuarina* and their PLOs provided strong evidence of cospeciation between host and endosymbiont. However, as the cophylogenetic relationship between *Neoparamoeba perurans* and their endosymbionts is yet to be assessed, it is uncertain whether the endosymbiotic relationship between all PLOs and their host for all species of *Neoparamoeba* is strictly monophyletic. In this study phylogenetic uncertainties in the relationship amongst *Neoparamoeba* were initially resolved by a secondary phylogenetic marker, the internal transcribed spacer 2 (ITS2). Supported by these new phylogenetic data, strict congruence in the phylogeny of all PLOs and their host *Neoparamoeba* was demonstrated implying that PLOs are most likely transmitted vertically from parent to daughter cell.



## 4.2 Introduction

Amoebae of the genus *Neoparamoeba* (Amoebozoa; Flabellinea; Dactylopodida; Vexilliferidae) are ubiquitous in the marine environments (Page, 1983; Page, 1987). They are small, lobose amoebae that form dactylopodiate pseudopodia in their locomotive form and are considered "naked" in that they lack cell-surface structures characteristic of other members of the Dactylopodida (Dyková et al., 2000; Dyková et al., 2005b; Page, 1983). The most discernable morphological feature of *Neoparamoeba*, shared only with *Paramoeba* and *Janickina* amoebae, is the possession of one or more intracellular, perinuclear endosymbiont (Dyková et al., 2003; Dyková et al., 2000). These endosymbionts were initially termed "parasomes" or "Nebenkörper" (Schaudinn, 1896) and are kinetoplastid-like, aflagellate, usually binucleated eukaryotic cells with a single, giant kinetoplastid-mitochondrion (Dyková et al., 2003). Morphological similarities between *Neoparamoeba* "parasomes" and *Perkinsiella amoebae*, an endosymbiont of the *Janickina* amoebae (Hollande, 1980) led to *Neoparamoeba* "parasomes" being named *Perkinsiella amoebae*-like organisms (PLOs) (Dyková et al., 2003; Moreira et al., 2004). Unfortunately the generic name *Perkinsiella* was already in use within another taxonomic group, therefore Dyková (2008) proposed renaming the generic name to *Perkinsela* which resolved the homonymy of its previous name whilst retaining the acronym PLO.

In most cases, when a host harbours an intracellular endosymbiont it profits from its biosynthetic capabilities (Moran et al., 2008). The advantages that *Neoparamoeba* and/or PLOs receive from their association are uncertain. However, as

*Neoparamoeba* trophozoites always retain at least one PLO in intimate association with their nucleus, their symbiotic relationship is assumed to be obligatory and classified as a mutualistic (Dyková et al., 2003; Dyková et al., 2008). Furthermore, based on the phylogenetic data that are available, a strong coevolutionary relationship exists between endosymbiotic PLOs and *Neoparamoeba pemaquidensis*, *Neoparamoeba branchiphila* and *Neoparamoeba aestuarina* (Caraguel et al., 2007b; Dyková et al., 2003; Dyková et al., 2008) suggesting that their symbiotic relationship is hereditary. Unfortunately the relationship between *Neoparamoeba perurans* and their PLOs is yet to be assessed. Since *N. perurans* represent one of the ancestral lineages of the *Neoparamoeba* group (Young et al., 2007) their addition to coevolutionary analyses is necessary in order to confirm whether a stable hereditary relationship exists amongst all *Neoparamoeba* and their endosymbiotic PLOs. In addition, *N. perurans* are distinct from other *Neoparamoeba* as they are the only pathogenic species and confirmed aetiological agents of amoebic gill disease (AGD) (Young et al., 2007; Young et al., 2008c). AGD can be fatal if left untreated and is a significant cause of mortality in marine fish reared under farm conditions (Dyková et al., 1995; Kent et al., 1988; Munday et al., 1990; Rodger and McArdle, 1996; Young et al., 2008c). It is unclear why only *N. perurans* elicits AGD even though other *Neoparamoeba* strains have been shown to cohabit the gills of AGD-affected fish (Dyková et al., 2005b; Dyková et al., 2007; Fiala and Dyková, 2003; Kent et al., 1988). Interestingly, *Neoparamoeba* are phylogenetically related to non-pathogenic, free-living amoebae (Peglar et al., 2003) whereas their endosymbiotic kinetoplastid PLOs are phylogenetically related to *Ichthyobodo* (Dyková et al., 2003; Moreira et al., 2004), known parasites of marine and freshwater fish (Grignard et al., 1996;

Urawa, 1995; Urawa et al., 1991). Determining the taxonomic position of endosymbionts of *N. perurans* in relation to PLOs of non-pathogenic strains of *Neoparamoeba* and pathogenic kinetoplastid organisms affecting marine and freshwater fish (Grignard et al., 1996; Lom, 1979; Urawa, 1995; Urawa et al., 1991; Woo, 1994; Woo, 2003) may offer insights into the role of *Neoparamoeba* endosymbionts in AGD pathogenesis.

Similarities in the trophozoite morphology amongst the *Neoparamoeba* species has led to taxonomic confusion, particularly in the context of disease aetiology (Dyková et al., 2000; Dyková et al., 2005b; Young et al., 2007). Therefore, definitive taxonomic classification of *Neoparamoeba* and their endosymbiotic PLOs is dependent on 18S ribosomal RNA gene based phylogenetic inference (Dyková et al., 2003; Dyková et al., 2008; Dyková et al., 2005b; Dyková et al., 2007; Fiala and Dyková, 2003; Steinum et al., 2008; Young et al., 2007). With the inclusion of new *Neoparamoeba* species and strains there is uncertainty concerning whether *N. perurans* or *N. branchiphila* represent the parental gene lineage (Young et al., 2007) and how the two morphologically distinct species, *Neoparamoeba pemaquidensis* and *Neoparamoeba aestuarina*, are related (Dyková et al., 2008; Steinum et al., 2008; Young et al., 2007). Additional phylogenetic markers were used to support the taxonomic classification of *Neoparamoeba perurans* (Young et al., 2007) and discriminate between strains of *N. pemaquidensis* (Caraguel et al., 2007b). Whilst being phylogenetically informative, these studies did not resolve the uncertainties that currently exist in *Neoparamoeba* phylogeny since representative strains from all *Neoparamoeba* species were not incorporated within the phylogenetic analyses.

Initially, the consequences of including two new *Neoparamoeba* strains to the 18S rRNA gene phylogeny was explored. Since phylogenetic uncertainties remained unresolved, we then examined the usefulness of the *Neoparamoeba* rRNA internal transcribed spacer region (ITS) as a secondary phylogenetic marker. The ITS2 was phylogenetically informative, separating *N. pemaquidensis* and *N. aestuarina* into distinct monophyletic clades and designating *N. perurans* as the most genetically divergent *Neoparamoeba* species. The new phylogenetic data were used to verify the tree topologies used in cophylogenetic analyses that revealed strict phylogenetic congruence between endosymbiotic PLOs with their host *Neoparamoeba*.

### **4.3 Materials and methods**

#### **4.3.1 Acquisition of clonal cultured and non-cultured gill-derived (NCGD) amoebae**

Two new strains of *Neoparamoeba* were collected for morphological and molecular characterisation (Table 4.1). Prior to their use in phylogenetic analyses, newly isolated strains and their endosymbionts, PLOs were characterised morphologically. Nomarski differential interference contrast (DIC) light microscopy was used to observe flattened trophozoites in hanging drop preparations. Clonal cultured strains of amoebae were obtained from culture collections held at the Biology Centre of the Academy of Sciences of the Czech Republic, Institute of Parasitology (České Budějovice, Czech Republic) and the National Centre for Marine Conservation and Resource Sustainability (NCMCRS), University of Tasmania (Launceston, Tasmania, Australia) (Table 4.1). Previous identification of strains was based on

phylogenetic analyses and 18S rRNA gene-specific PCR (Dyková et al., 2003; Dyková et al., 2008; Dyková et al., 2007; Dyková et al., 2005c). Primary isolation, purification, culture and harvesting of trophozoites were performed following methods described previously (Dyková et al., 2000; Dyková et al., 2005b).

#### **4.3.2 DNA extraction and PCR amplification of rRNA genes from host amoeba and endosymbiont**

Genomic DNA from *Neoparamoeba* strains was prepared as previously described (Dyková et al., 2003; Fiala and Dyková, 2003; Young et al., 2007). Genomic DNA of non-cultured gill-derived (NCGD) amoebae from AGD-affected Atlantic salmon was obtained in a previous study (GD-D1-1, GD-D1-2 and GD-D1-3) where the amoebae were identified as *N. perurans* (Young et al., 2007). The same DNA templates that were used to PCR amplify the full-length 18S rRNA gene of *Neoparamoeba* were also used to PCR amplify the partial 18S rRNA gene of their PLOs as previously described (Dyková et al., 2008; Dyková et al., 2005b). The PCR amplification of genomic rRNA from *Neoparamoeba* including a partial 3' region of the 18S rRNA gene, internal transcribed spacer 1 (ITS1), the 5.8S rRNA gene, internal transcribed spacer 2 (ITS2) and a partial 5' region of the 28S rRNA gene was performed as previously described (Dyková et al., 2005a). The cloning of gene products and purification of plasmid DNA was also performed as previously described (Fiala and Dyková, 2003).

Table 4.1. *Neoparamoeba* strains used in this study.

<i>Neoparamoeba</i> species	Host amoeba		PLOs <sup>b</sup>		Host species	Location <sup>d</sup>	Reference <sup>e</sup>
	Amoeba strain/clone	18S rRNA gene <sup>a</sup>	ITS <sup>a</sup>	18S rRNA gene <sup>a</sup>			
<i>N. pemaquidensis</i>	AFSM11		1		<i>Psetta maxima</i>	Gill, NW Spain	(Fiala and Dyková, 2003)
	ASL1	1	1	1	<i>Salmo salar</i>	Gill, Launceston, Tasmania, Australia	<i>This manuscript</i>
	FRS/I		1		<i>S. salar</i>	Gill, Launceston, Tasmania, Australia	(Dyková et al., 2005b)
	GILLNOR1/I		1		<i>S. salar</i>	Gill, D'Entrecasteaux Channel, Bruny Island, Tasmania, Australia	(Dyková et al., 2005b)
	GILLNOR2/I		1		<i>S. salar</i>	Gill, D'Entrecasteaux Channel, Bruny Island, Tasmania, Australia	(Dyková et al., 2005b)
	GILLRICH3/I		1		<i>S. salar</i>	Gill, Launceston, Tasmania, Australia	(Dyková et al., 2007)
	LITHON		1		<i>Lithophyllum racemes</i>	Surface of algae, location unknown	(Dyková et al., 2008)
	NET12AFL/I		1		n/a	Marine cage net material, Huon Estuary, Tasmania, Australia	(Dyková et al., 2007)
	NETC1/I		1		n/a	Marine cage net material, Huon Estuary, Tasmania, Australia	(Dyková et al., 2005b)
	NETC2/I		1		n/a	Marine cage net material, Huon Estuary, Tasmania, Australia	(Dyková et al., 2005b)
	NETH2T3		1		n/a	Marine cage net material, Huon Estuary, Tasmania, Australia	(Dyková et al., 2005b)
	NP251002/I		3		<i>S. salar</i>	Gill, Launceston, Tasmania, Australia	(Dyková et al., 2005b)
	PA027/I		1		<i>S. salar</i>	Gill, Dover, Tasmania, Australia	(Dyková et al., 2005b)
	PAL2	1	1	1	<i>Palmaria palmate</i>	Surface of algae, Trondheim, Vevang, Norway	<i>This manuscript</i>
	SED5A/I		1		n/a	Sediments, Wedge Bay, Tasman Peninsula, Tasmania, Australia	(Dyková et al., 2005b)
	SEDC1/I		1		n/a	Sediments, Bicheno, Tasmania, Australia	(Dyková et al., 2005b)
	SEDCB1/I		1		n/a	Sediments, Bicheno, Tasmania, Australia	(Dyková et al., 2005b)
	SEDCT1/I		1		n/a	Sediments, Tamar Estuary, Tasmania, Australia	(Dyková et al., 2005b)
	SEDST1/I		1		n/a	Sediments, Stringers Cove, Tasman Peninsula, Tasmania, Australia	(Dyková et al., 2005b)
	ST8V/I		1		<i>S. salar</i>	Gill, Dover, Tasmania, Australia	(Dyková et al., 2005b)
	WT2708/I		3		<i>S. salar</i>	Gill, Launceston, Tasmania, Australia	(Dyková et al., 2007)
	WTUTS/I		1		<i>S. salar</i>	Gill, Launceston, Tasmania, Australia	(Dyková et al., 2005b)
<i>N. aestuarina</i>	SU03		3		<i>Sphaerechinus granularis</i>	Radial water vessels and side branches, location unknown	(Dyková et al., 2008)
<i>N. branchiphila</i>	AFSM3		1		<i>P. maxima</i>	Gill, NW Spain	(Fiala and Dyková, 2003)
	AMOP1		1		<i>Paracentrotus lividus</i>	Cretan Sea, Kápathos Island, Greece	(Dyková et al., 2007)
	NRSS/II		3		<i>S. salar</i>	Gill, Launceston, Tasmania, Australia	(Dyková et al., 2005b)
	SEDMH1/I		1		n/a	Sediments, Macquarie Harbour, Tasmania, Australia	(Dyková et al., 2005b)
	SM53		1		<i>P. maxima</i>	Gill, NW Spain	(Fiala and Dyková, 2003)
	SM68		1		<i>P. maxima</i>	Gill, NW Spain	(Fiala and Dyková, 2003)
	SU4		1		<i>Helicodiaris erythrogramma</i>	Tamar River, Georgetown, Tasmania, Australia	(Dyková et al., 2007)
	TG1267		1		<i>Thunnus maccoyii</i>	Gill, Port Lincoln, South Australia, Australia	(Dyková et al., 2007)
	GD-D1 1		1 <sup>c</sup>	1 <sup>c</sup>	<i>S. salar</i>	Gill, Launceston, Tasmania, Australia	(Young et al., 2007)
	GD-D1 2		1 <sup>c</sup>	1 <sup>c</sup>	<i>S. salar</i>	Gill, Launceston, Tasmania, Australia	(Young et al., 2007)
<i>N. perurans</i>	GD-D1 3		3 <sup>c</sup>	1 <sup>c</sup>	<i>S. salar</i>	Gill, Launceston, Tasmania, Australia	(Young et al., 2007)

<sup>a</sup> Number of gene sequences PCR-amplified from a single clonal cultured *Neoparamoeba* strain

<sup>b</sup> *Perkinsela amoebae*-like organisms (PLOs)

<sup>c</sup> Gene sequences derived from primary amoebae isolates since *N. perurans* cannot be maintained in culture

<sup>d</sup> The tissue and geographical location from which amoebae were isolated

<sup>e</sup> Original reference that described the *Neoparamoeba* strain

Nucleotide sequencing was performed using ABI Prism BigDye Terminator (version 3.1) Cycle Sequencing Kit (Applied Biosystems (ABI), Scoresby, Victoria, Australia) following the manufacturer's instructions. Sequencing reactions were initiated using plasmid DNA template and the insert was amplified using M13 forward or reverse oligonucleotides. Samples were analysed on an ABI 3730xl DNA analyser (ABI). The 18S rRNA gene and ITS nucleotide sequences analysed in this study were deposited in GenBank (National Centre for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD) and assigned the accession numbers shown in Figures 4.2, 4.3, 4.4 and 4.5.

#### **4.3.3 ITS1 and ITS2 secondary structure**

The ITS1 and ITS2 nucleotide sequences of 34 *Neoparamoeba* strains, 5 of which had three replicates per clonal cultured strain, were folded separately using the default settings in RNAstructure, version 4.6 (Mathews et al., 2004). ITS nucleotide sequences were then homology-based modelled against ITS secondary structures until representative structures with more than 75% transfer of each helix were obtained at <http://its2-2.bioapps.biozentrum.uni-wuerzburg.de/cgi-bin/index.pl?custom> using criteria previously described (Wolf et al., 2005). Final two-dimensional plots of secondary structure were produced using RNAdraw, version 1.1 (Matzura and Wennborg, 1996).

#### **4.3.4 Construction and support for *Neoparamoeba* and PLO phylogeny using the 18S rRNA gene and the ITS**

The 18S rRNA gene sequences from the new *Neoparamoeba* strains PAL2 and ASL1, 58 strains of *Neoparamoeba* obtained from GenBank and an outgroup containing *Korotnevella stella* (GenBank accession number: AY183893), *Korotnevella hemistylepis* (AY121850), *Vexillifera armata* (AY183891), *Vannella anglica* (AF099101) and *Vannella aberdonica* (AY121853) were aligned in ClustalX (Thompson et al., 1997) (gap opening/gap extension penalty = 8/1). The alignment was checked manually then interrogated using semi-strict block parameter settings (Table 4.2) in Gblocks 0.91 (Castresana, 2000) to remove poorly-aligned gene regions prior to phylogenetic analyses.

Phylogenetic tree searches using the aligned *Neoparamoeba* 18S rRNA gene sequences were conducted using three methods of analysis. Maximum parsimony analyses (MP) were performed in PAUP\*4 (Swofford, D.L. PAUP\*: phylogenetic analysis using parsimony version 4.0b10. 2001. Sunderland, MA, Sinauer Assoc.) using a heuristic search with tree-bisection-reconnection (TBR) branch swapping, a transition/transversion ratio of 1:2 and 10 random-taxon addition iterations. Gaps were treated as missing data. Clade reliability was estimated by implementing bootstrap resampling (500 replicates) in PAUP using a heuristic search with TBR branch swapping and transition/transversion ratio of 1:2.



Table 4.2. Akaike information criteria test-selected parameters in Modeltest version 3.7 (Posada and Crandall, 1998) maximum likelihood models and subsequently applied to phylogenetic analyses.

Nucleotide sequence	Gblocks parameters	Evolutionary model	Alpha parameter	Proportion of invariable sites	T ratio	Frequency of base proportions <sup>g</sup>	Substitution rate model matrix <sup>h</sup>
<i>Neoparamoeba</i> 18S rRNA gene	34 <sup>a</sup> , 56 <sup>b</sup> , 8 <sup>c</sup> , 10 <sup>d</sup> , with half <sup>e</sup> , 85% <sup>f</sup>	GTR+I+G	0.474	0.3497	n/a	0.29, 0.16, 0.23, 0.33	1.22, 8.24, 2.10, 0.38, 8.92, 1.00
<i>Neoparamoeba</i> 18S rRNA gene selected <sup>*</sup>	23, 37, 8, 10, with half, 100%	GTR+I+G	0.511	0.3357	n/a	0.29, 0.15, 0.23, 0.33	1.14, 6.77, 1.99, 0.16, 8.11, 1.00
<i>Perkinsela amoeba</i> -like organism 18S rRNA gene	22, 35, 8, 10, with half, 85%	TrN+I+G	0.765	0.4294	n/a	0.24, 0.24, 0.30, 0.22	1.00, 2.14, 1.00, 1.00, 3.83, 1.00
<i>Neoparamoeba</i> ITS2	27,27,8,2, with half, 44%	HKY+G	0.7991	0	1.0161	0.35,0.15,0.11,0.39	Not fixed

<sup>\*</sup> *Neoparamoeba* 18S rRNA gene sequences used for host/endosymbiont coevolution analyses

Gblocks parameters: minimum number of nucleotide sequences for a conserved position<sup>a</sup>, minimum number of nucleotide sequences for a flanking position<sup>b</sup>, maximum number of contiguous non-conserved positions<sup>c</sup>, minimum length of a block<sup>d</sup> and allowed gap positions<sup>e</sup>

<sup>f</sup> Proportion of aligned nucleotide sequences retained after gBLOCKS analyses

<sup>g</sup> Frequency of base proportions (A, C, G,T)

<sup>h</sup> Substitution rate model matrix ([A–C], [A–G], [A–T], [C–G], [C–T],[G–T])

The Akaike information criteria test in Modeltest version 3.7 (Posada and Crandall, 1998) selected the most appropriate likelihood model for further phylogenetic analyses (Table 4.2). Tree searches by maximum likelihood (ML) analysis were performed in PAUP\*4 (Swofford, 2001) using the heuristic process with TBR branch swapping and 10 random-taxon addition iterations. Clade reliability for the most parsimonious ML tree was estimated using bootstrap resampling with 500 replicates generated by SEQBOOT in PHYLIP Version 3.66 (Felsenstein, 1989). ML analysis of the 500 bootstrap replicates was performed with PHYML (Guindon and Gascuel, 2003) with the model settings previously specified (Table 4.2) and using the ML tree obtained in PAUP\*4 as the starting tree. Bayesian phylogenetic inference (BI) was determined using Markov chain Monte Carlo (MCMC) analysis in MrBayes, version 3.1.2 (Huelsenbeck and Ronquist, 2005; Ronquist and Huelsenbeck, 2003). The likelihood parameters were set as described above and four million generations of MCMC analysis were performed with trees recorded every 100th generation. At this point, the standard deviation of split frequencies was <0.01 and the potential scale reduction factor approached one. Branch support for each tree was generated using the 50% majority rule criterion on bootstrap replicate trees generated with MP and ML analyses and the final 75% of trees generated by BI.

The 18S rRNA gene sequences from the PLOs of clonal cultured strains PAL2 and ASL1, three independent NCGD amoebae primary isolates (GD-D1-1, GD-D1-2 and GD-D1-3), 34 strains of *Neoparamoeba* with 18S rRNA gene sequences obtained from GenBank and an outgroup containing *Ichthyobodo necator* (GenBank accession number: AY224691), *I. hippoglossi* (DQ414520) and *Ichthyobodo* sp. HC-2003

(AY297477) were aligned in ClustalX (Thompson et al., 1997) (gap opening/gap extension penalty = 8/1). The nucleotide sequence alignment was checked manually then interrogated using semi-strict block parameter settings (Table 4.2) in Gblocks 0.91. Phylogenetic analyses of the 18S rRNA gene sequences from the PLOs were as described above with model parameters specified in Table 4.2. Phylogenetic analyses were also performed on selected *Neoparamoeba* 18S rRNA gene sequences from which the 18S rRNA gene sequences of the PLO were available. Analysis methods were as described above with model parameters specified (Table 4.2).

5.8S rRNA gene sequences were obtained from 31 clonal culture strains of *Neoparamoeba* (Table 4.1), three independent NCGD amoebae primary isolates (GD-D1-1, GD-D1-2 and GD-D1-3) and 14 gene sequences from strains of *Neoparamoeba* obtained from GenBank. In addition, three replicate nucleotide sequences were obtained from four clonal culture strains of *Neoparamoeba* (Table 4.1), one NCGD amoebae primary isolate (GD-D1-3) and three clonal culture strains of *N. pemaquidensis* obtained from GenBank. All 5.8S rRNA gene sequences were aligned in ClustalX (Thompson et al., 1997) (gap opening/gap extension penalty = 8/1) and checked manually. Genetic distances amongst *Neoparamoeba* 5.8S rRNA gene sequences were calculated as mean character differences using PAUP\*4.

The ITS2 nucleotide sequences from the same amoebae strains described for the 5.8S rRNA gene were aligned using their nucleotide bases and secondary structures in 4SALE, version 0.995 (Seibel et al., 2006) with an ITS2-specific substitution matrix. Phylogenetic analyses of the ITS2 nucleotide sequences from strains of *N.*

*pemaquidensis*, *N. aestuarina* and *N. branchiphila* were performed using the methods described above and the model parameters specified in Table 4.2.

#### **4.3.5 Cophylogenetic analyses of *Neoparamoeba* and their PLO endosymbiont**

Coevolution of the host *Neoparamoeba* and the endosymbiotic PLO was assessed using the DistCoA and subsequent ParaFit analyses in CopyCat, version 1.00.13 (Meier-Kolthoff et al., 2007). A *Neoparamoeba* and a PLO distance matrix was derived from the maximum likelihood trees produced using PAUP\*4. These matrices and a matrix including the 39 *Neoparamoeba*-PLO associations were imported into CopyCat. CopyCat invoked DistPCoA to compute eigenvectors (discarding those with negative values) as well as ParaFit using 9,999 random permutations to test whether the host *Neoparamoeba* sequences associated randomly with each PLO sequence or according to the genetic distances estimated for the two groups of organisms.

### **4.4 Results**

#### **4.4.1 Trophozoite morphology and taxonomic classification of two new amoebae strains**

Two new strains of *Neoparamoeba* were isolated and clonal cultured, one from the surface of an alga, *Palmaria palmata* (Fig. 4.1A: PAL2) and the other from AGD-affected Atlantic salmon gills (Fig. 4.1B: ASL1). Consistent with previous descriptions of *Neoparamoeba*, the morphology of representative trophozoites of either strain were indistinguishable (Dyková et al., 2000; Dyková et al., 2005b; Dyková et al., 2007).

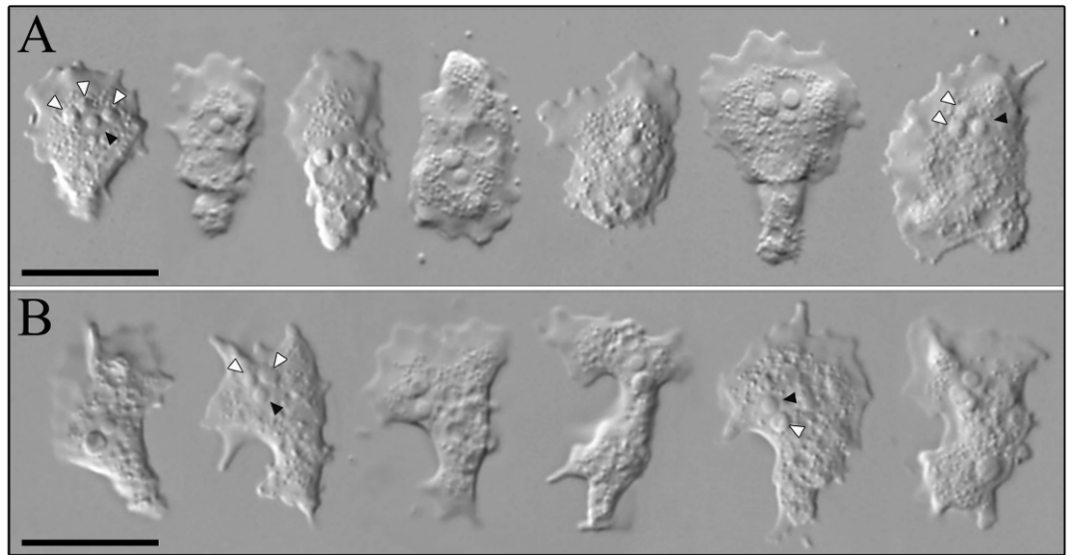


Figure 4.1. Morphology of *Neoparamoeba* strains isolated and clonal cultured from the surface of a red alga, *Palmaria palmata* (A: PAL2) and Atlantic salmon, *Salmo salar* gill tissue (B: ASL1). *Perkinsela amoebae*-like organisms are present (white arrow heads) adjacent to the nucleus (black arrow heads) of host amoeba. Scale bars represent 20  $\mu\text{m}$ .

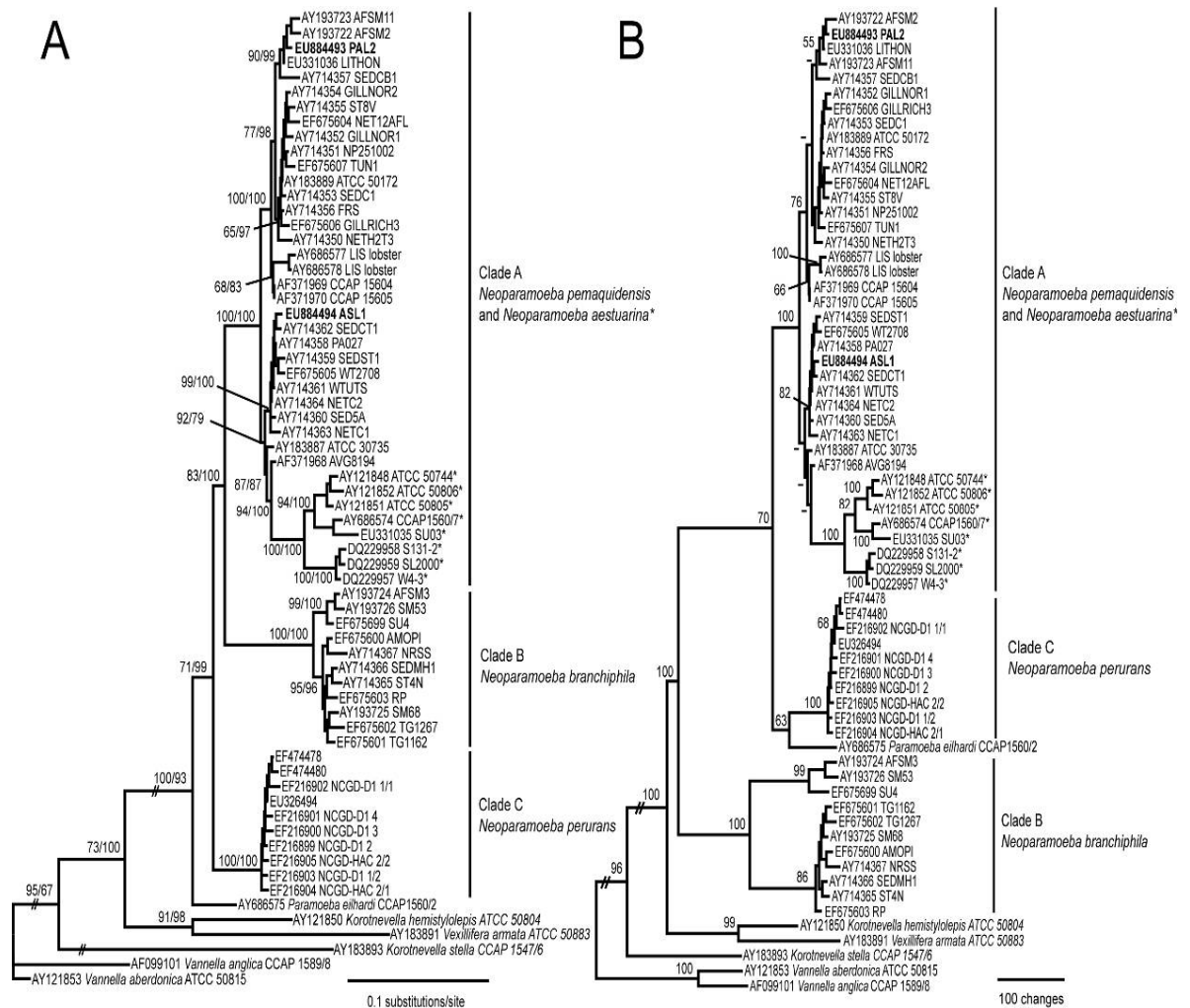


Figure 4.2. Based on the 18S rRNA gene phylogeny two new clonal cultured *Neoparamoeba* strains cluster with the *Neoparamoeba pemaquidensis*, however the overall tree topology and the phylogenetic relationship between *N. pemaquidensis* and *Neoparamoeba aestuarina* remain unresolved. A. Maximum likelihood tree ( $-\ln = 11638.86$ ) resulting from the phylogenetic analysis of *Neoparamoeba* 18S rRNA gene sequences incorporating newly isolated strains and nucleotide sequences recently deposited in GenBank. Phylogeny of *Neoparamoeba* was inferred from the 18S rRNA gene sequences using maximum likelihood (ML) and Bayesian phylogenetic inference (BI). BI and ML analyses yielded equally parsimonious trees that distinguished three *Neoparamoeba* clades. Clade A is composed of strains of *N. pemaquidensis* and *N. aestuarina* (denoted by an asterisk). Clade B is composed of strains of *Neoparamoeba branchiphila*. Clade C is composed of 18S rRNA gene sequences of *Neoparamoeba perurans*. B. Most parsimonious tree resulting from maximum parsimony phylogenetic analysis of *Neoparamoeba* 18S rRNA gene sequences. Monophyletic clades were in agreement with the other phylogenetic analyses, however the trees differed in topology. New nucleotide sequences are in bold. Values indicated on the branches represent >50% bootstrap support (A: ML/BI) and dashes denote <50% bootstrap support. The GenBank accession number and strain identification of each nucleotide sequence is shown.

Trophozoites contained one or more perinuclear PLOs (Fig. 4.1, white arrow) (Dyková et al., 2000). Each strain was larger than the conspicuously smaller trophozoites of *N. aestuarina* (Dyková et al., 2008). To discriminate between the remaining morphologically similar *Neoparamoeba* species the taxonomic positions of PAL2 and ASL1 were inferred from their 18S rRNA gene phylogeny. The *Neoparamoeba* 18S rRNA gene alignment consisted of 2205 nucleotide sites, and of these, 1871 (85%) were retained (Table 4.2) and 713 were parsimony-informative. The ML and BI analyses yielded three distinct lineages within the *Neoparamoeba* genus (rooted ML tree, clades A, B, & C. Fig. 4.2A). Strains of *N. pemaquidensis* and *N. aestuarina* were unable to be separated and clustered within a large monophyletic group (clade A). There was strong support for *N. branchiphila* strains (clade B, 83-100%) and *N. perurans* (clade C, 71-99%) as two distinct monophyletic groups. The MP analysis of the *Neoparamoeba* 18S rRNA gene sequence alignment yielded a tree that was similar to the BI and ML analysis (Fig. 4.2B). Overall support for the three distinct lineages within the *Neoparamoeba* genus was maintained, however strains of *N. branchiphila* formed a monophyletic group (clade B, 100% support) positioned as the more divergent of the *Neoparamoeba*. Additionally, strains of *N. perurans* were still phylogenetically distinguishable and formed a monophyletic group (clade C, 63% support) most similar to the putative *Paramoeba eilhardi* isolate. The two *Neoparamoeba* strains sequenced in this study (Fig. 4.2A & B, bold text) fell within the *N. pemaquidensis*/*N. aestuarina* clade, most similar in 18S rRNA gene phylogeny to *N. pemaquidensis*. The inclusion of sequences belonging to new clonal cultured strains of *Neoparamoeba* and primary isolates of *N.*

*perurans* from Atlantic salmon cultured in Norway within the 18S rRNA phylogenetic tree did not resolve the phylogenetic relationship between *N. pemaquidensis* and *N. aestuarina*. Therefore, the usefulness of the ITS as an additional phylogenetic marker to discriminate between *N. pemaquidensis* and *N. aestuarina* was assessed.

#### **4.4.2 Analysis of the ITS and its support for the proposed *Neoparamoeba* phylogenetic lineages**

The *Neoparamoeba* ITS, consisting of the 5.8S rRNA gene, ITS1 and ITS2 was assessed as a phylogenetic marker. Since the ITS1, ITS2 and 5.8S rRNA gene regions are subject to different evolutionary pressures (Álvarez and Wendel, 2003), each region has a different level of nucleotide sequence variability. Therefore, each region was analysed separately to apply the correct evolutionary models during phylogenetic analyses. The nucleotide similarity values among the 153-154 bp 5.8S rRNA gene sequences are summarised as the range of percentage identities among the phylogenetic lineages examined (Table 4.3). The 5.8S rRNA gene was useful for confirming that the ITS from NCGD amoebae isolates was derived from *N. perurans* and not from other possible contaminating eukaryotic genomic DNA. However, the 5.8S rRNA gene was highly conserved across all *Neoparamoeba* and therefore was not phylogenetically informative (Table 4.3). Conversely, the ITS1 was highly variable within and between the *Neoparamoeba* phylogenetic lineages with the length of *N. perurans* (308-314 bp), *N. pemaquidensis* (240- 261 bp), *N. branchiphila* (234-251 bp) and *N. aestuarina* (236-251 bp) ITS1 providing evidence of insertion and deletion events. No conserved secondary structure could be elucidated for the ITS1 and consequently no reliable nucleotide sequence alignment could be obtained (data not shown).



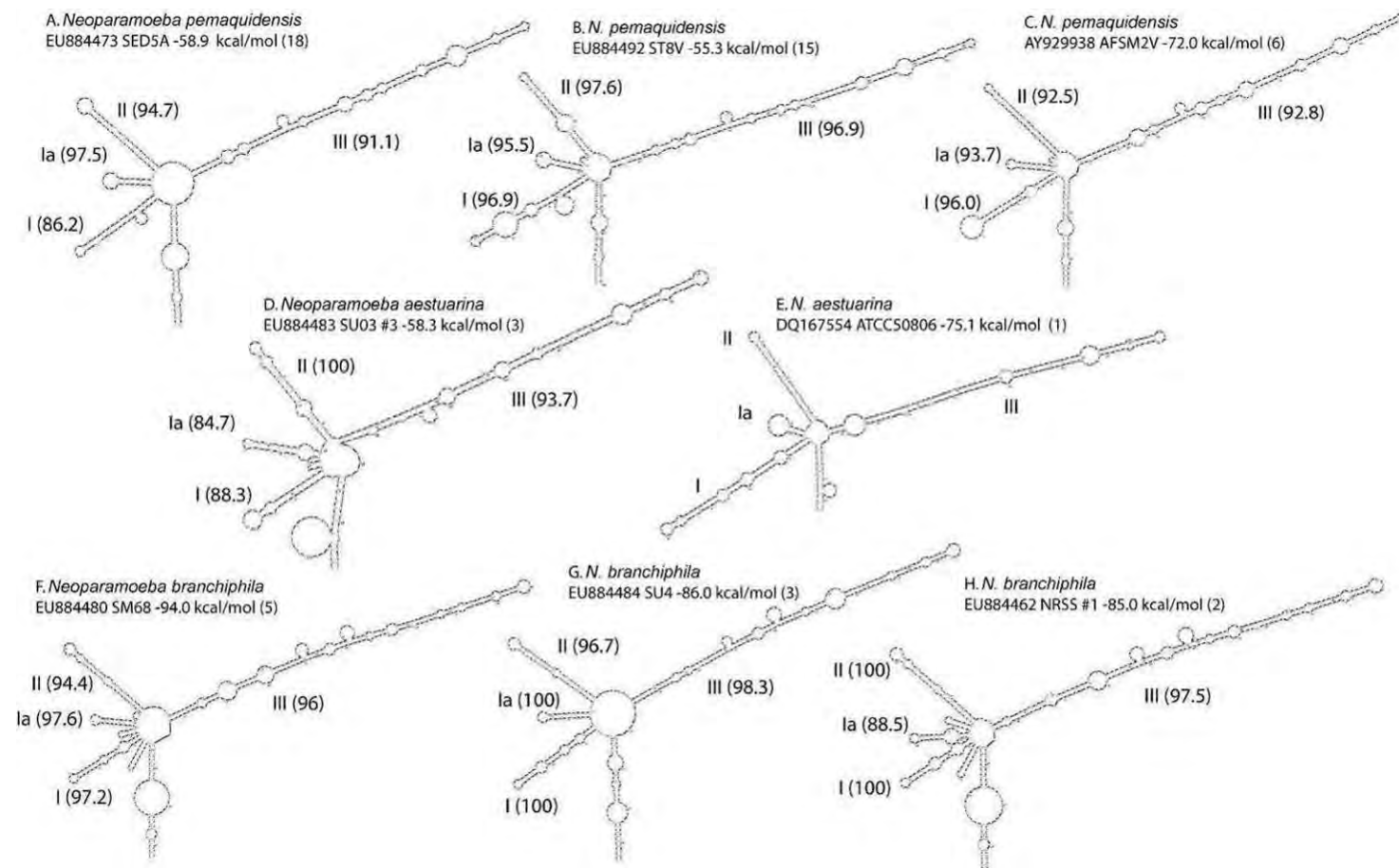


Figure 4.3. *Neoparamoeba pemaquidensis*, *N. branchiphila* and *N. aestuarina* retain a common ribosomal RNA internal transcribed spacer 2 (ITS2) secondary structure. Graphical visualisation (2D-plot) of the predicted ITS2 secondary structure of representative *Neoparamoeba* strains. The GenBank accession number and RNA-fold free energy state are displayed for each representative structure. The number of ITS2 nucleotide sequences predicted to conform (>75% transfer) to each secondary structure is summarised (parentheses) and each ITS2 nucleotide sequence is displayed with its secondary structure in Figure 4.4 (letters A to H). Each helix (numbered I to III) is displayed with the average percentage helix transfer (parentheses) when more than one nucleotide sequence is represented by the structure.

Table 4.3. Similarity among the aligned *Neoparamoeba* nucleotide sequences derived from mean character differences (percent).

Gene	Species	<i>N. pemaquidensis</i>			<i>N. aestuarina</i>	<i>N. branchiphila</i>	<i>N. perurans</i>
	Region	All	Clade A <sup>b</sup>	Clade B <sup>b</sup>			
5.8S rRNA	<i>Neoparamoeba pemaquidensis</i>	96.7-100			97.4-100	96.1-100	96.1-100
	<i>N. aestuarina</i>				97.4-100	97.4-100	97.4-100
	<i>N. branchiphila</i>					96.1-100	96.1-100
	<i>N. perurans</i>						96.1-100
ITS2	<i>N. pemaquidensis</i> intragenomic	93.5-97.6 (5) <sup>a</sup>	93.5-97.1 (2)	93.5-96.6 (2)	-	-	-
	<i>N. aestuarina</i> intragenomic				91.5-93.5 (1)	-	-
	<i>N. branchiphila</i> intragenomic					93.3-95.4 (1)	-
	<i>N. perurans</i> intra-/intergenomic <sup>c</sup>						97.4-99.4 (1)
	<i>N. pemaquidensis</i> (all)	72.8-99.7	-	-	56.1-65.0	45.8-52.3	-
	<i>N. pemaquidensis</i> (clade A) <sup>b</sup>		77.4-99.7	72.8-79.8	56.1-59.8	45.8-51.3	-
	<i>N. pemaquidensis</i> (clade B) <sup>b</sup>			84.2-97.2	60.9-65.0	47.8-52.3	-
	<i>N. aestuarina</i>				66.1-93.5	42.8-46.3	-
	<i>N. branchiphila</i>					70.4-96.8	-

<sup>a</sup> Displayed in parentheses is the number of clonal cultured *Neoparamoeba* strains used to assess intragenomic variability

<sup>b</sup> *N. pemaquidensis* strains were divided into two monophyletic clades based on the ITS2 phylogeny (see Fig. 4.4)

<sup>c</sup> Nucleotide sequences were derived from primary isolates of *N. perurans* and therefore gene variability is both intra- and intergenomic.

Therefore, the *Neoparamoeba* ITS1 was not subjected to phylogenetic analyses. Structural homology modelling of the ITS2 nucleotide sequences from *Neoparamoeba* produced conserved secondary structures for *N. pemaquidensis* (Fig. 4.3A-C), *N. aestuarina* (Fig 4.3D & E) and *N. branchiphila* (Fig. 4.3F-H) that were similar to the predicted common core of the ITS2 secondary structure identified throughout the Eukaryota (Coleman, 2003; Coleman, 2007; Schultz et al., 2005). The secondary structure of *N. perurans* ITS2 could not be predicted due to the differences in their nucleotide sequences (data not shown) which indicated that they are distinct from all the other *Neoparamoeba* species. This supports the phylogeny inferred from the ML derived 18S rRNA gene phylogenetic analysis (Fig. 4.2A) positioning *N. perurans* as the most divergent of the *Neoparamoeba* species. The RNA-fold free energy state of the representative ITS2 secondary structures are displayed in addition to the average percentage of each helix that was transferable from all the ITS2 nucleotide sequences (Fig. 4.3). Three of the four predicted helices are retained with helix III being the longest. An additional helix (helix Ia) is predicted among the *Neoparamoeba* rRNA ITS2 nucleotide sequences. The unique secondary structure of *N. perurans* ITS2 excluded this species from subsequent phylogenetic analyses since the predicted secondary structure of the *Neoparamoeba* ITS2 (Table 4.3) was used to drive their nucleotide sequence alignment. Of the 566 nucleotide sites, 254 (45%) were retained (Table 4.2) and 162 were parsimony-informative. The MP, ML and BI analyses yielded four distinct lineages within the *Neoparamoeba* (ML tree, clades A, B, C and D Fig. 4.4). Strains of *N. pemaquidensis*, *N. aestuarina* and *N. branchiphila* clustered within monophyletic clades. Furthermore, the *N. pemaquidensis* could be sub-divided into two monophyletic clades (clade A and B, Fig. 4.4). There was strong support for the

separation of *N. pemaquidensis* strains (clade A and B, 75-92% support) and *N. aestuarina* (clade C, 100% support) as distinct monophyletic groups with *N. aestuarina* positioned between *N. pemaquidensis* and *N. branchiphila* as previously described (Dyková et al., 2005b; Fiala and Dyková, 2003). In most cases, the predicted ITS2 secondary structures of each *Neoparamoeba* strain (Fig. 4.3A-H) clustered within their ITS2-based phylogenetic clades (Fig. 4.4, denoted by the letters A-H next to the strain identifier).

Replicate ITS2 nucleotide sequences from the same clonal cultured strains of *Neoparamoeba* clustered together within their respective phylogenetic lineages despite displaying substantial intragenomic heterogeneity (Table 4.3). Therefore intragenomic variability did not significantly contribute to the overall tree topology. Interestingly, the lowest ITS2 nucleotide sequence variability was observed within the *N. perurans* isolates (Table 4.3), which were not clonal cultured and their sequences could be representative of inter- and intragenomic heterogeneity.

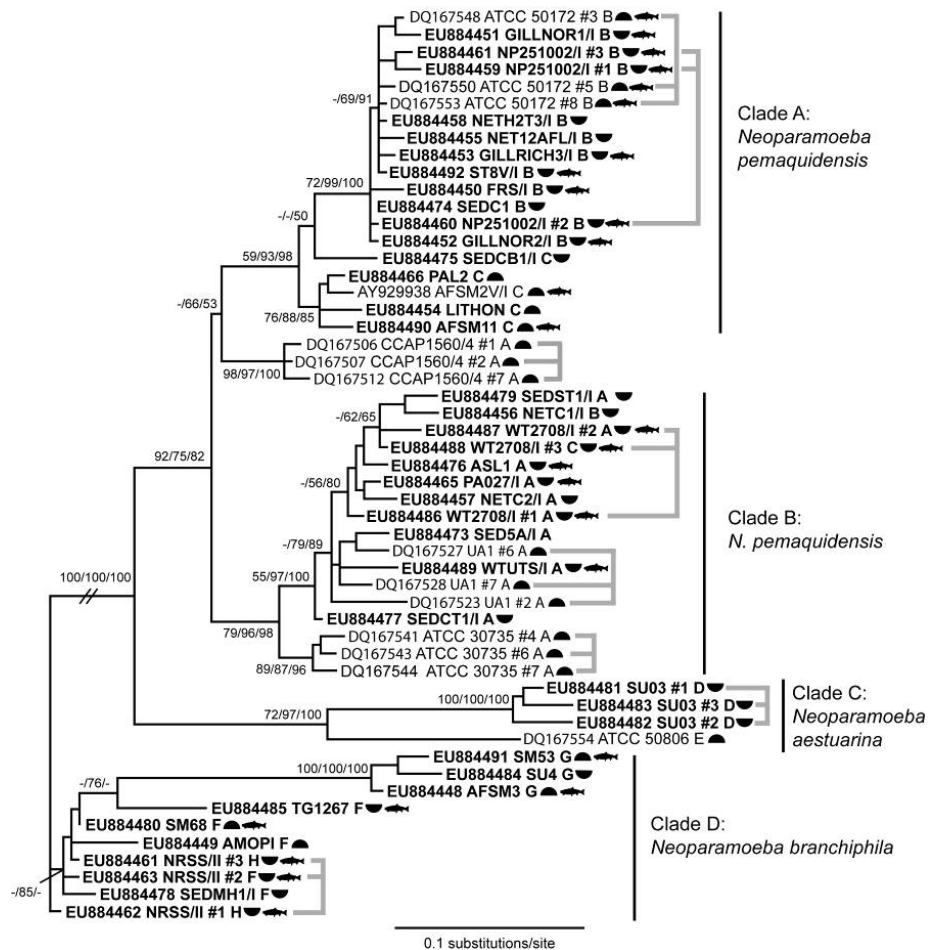


Figure 4.4. The phylogeny inferred from the internal transcribed spacer 2 (ITS2) resolves the taxonomic relationship between *Neoparamoeba pemaquidensis* and *Neoparamoeba aestuarina*. Maximum likelihood tree ( $-\ln = 2240.06$ ) resulting from the phylogenetic analysis of *Neoparamoeba* ITS2 nucleotide sequences aligned using secondary structure. Phylogeny of *Neoparamoeba* was inferred from the ITS2 nucleotide sequences using maximum parsimony (MP), maximum likelihood (ML) and Bayesian phylogenetic inference (BI). Analyses yielded equally parsimonious trees that distinguished four *Neoparamoeba* clades. Clade A and B are composed of strains of *Neoparamoeba pemaquidensis*. Clade C is composed of strains of *Neoparamoeba aestuarina* and Clade D is composed of strains of *Neoparamoeba branchiphila*. New nucleotide sequences are in bold. Values indicated on the branches represent >50% bootstrap support (MP/ML/BI) and dashes denote <50% bootstrap support. The GenBank accession number of each nucleotide sequence is shown. The letters A to H denote their representative ITS2 secondary structures corresponding to those shown in Figure 4.3. Nucleotide sequences connected by grey lines are replicate sequences from the same *Neoparamoeba* strain. The geographical location of *Neoparamoeba* strains is denoted by a semi-circle, representing the southern (☉) and northern (☿) hemisphere. Amoebae strains isolated and clonal cultured from the gills of fish are highlighted (fish symbol). New nucleotide sequences are in bold.

Based on the ITS2 phylogeny, the two new *Neoparamoeba* isolates characterised in this study aligned within the *N. pemaquidensis* clades. The clonal culture strain isolated from the alga, *P. palmate* (PAL2) aligned with *N. pemaquidensis* (clade A), closely related to strains isolated from the algae, *L. racemes* (LITHON) and the gills of turbot (AFSM11 and AFSM2V). The clonal culture strain isolated from the gills of Atlantic salmon (ASL1) aligned with *N. pemaquidensis* (clade B), closely related to strains isolated from Atlantic salmon (WT2708 and PA027) or from sediment samples taken from the vicinity of Atlantic salmon sea-cages (SEDST1, NETC1 and NETC2). *Neoparamoeba* strains or species did not cluster within monophyletic groups in the context of their initial isolation from either the northern and southern hemispheres nor did strains of *N. pemaquidensis* and *N. branchiphila* group based on their isolation from marine fish (Fig. 4.4).

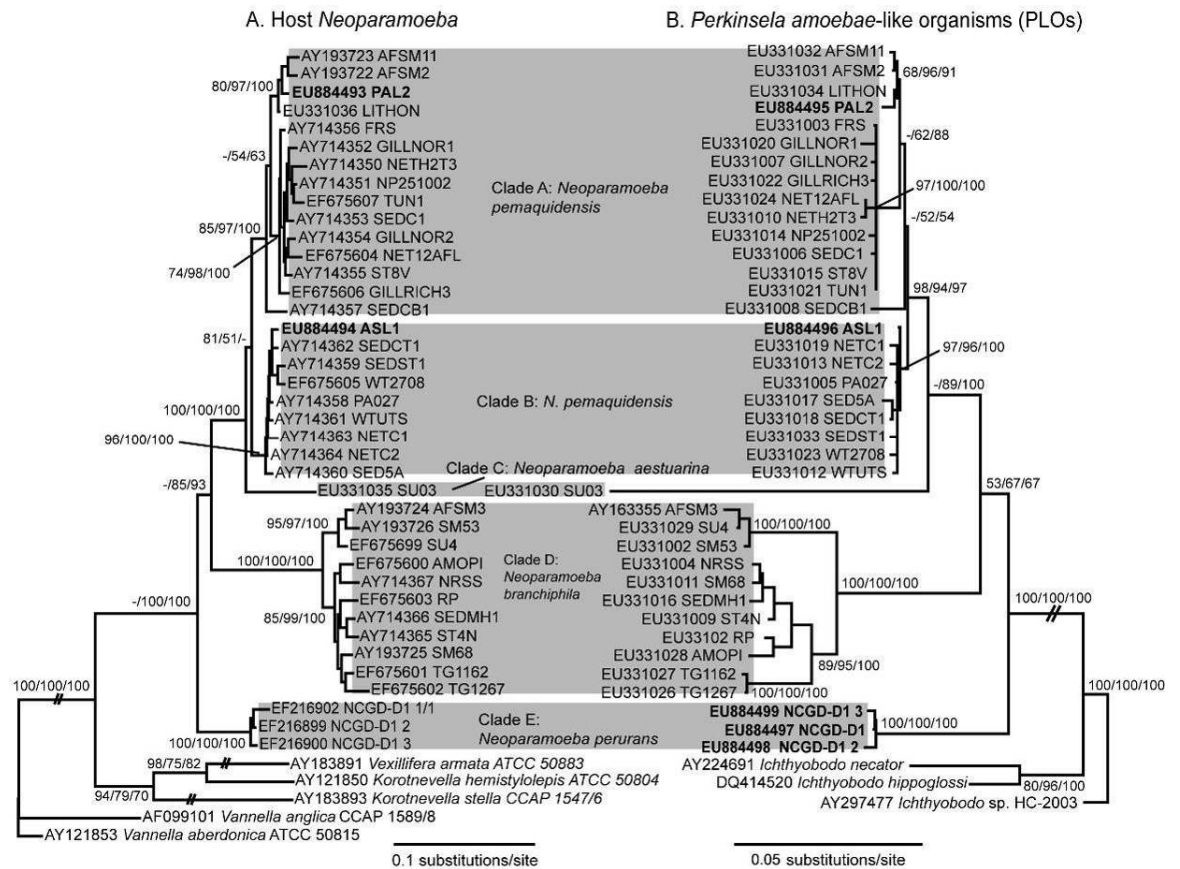


Figure 4.5. Strict coevolution of *Neoparamoeba* and their endosymbionts, *Perkinsela amoebae*-like organisms (PLOs), implies that a single event in an ancestral species led to the symbiotic relationship. Phylogenetic analyses of the 18S rRNA genes of host *Neoparamoeba* and their endosymbionts supports strict coevolution. Maximum likelihood trees inferred from the 18S rRNA genes of *Neoparamoeba* (A,  $-\ln = 12015.76$ ) and their endosymbionts, *Perkinsela amoebae*-like organisms (B,  $-\ln = 5865.71$ ). Phylogenetic clades inferred from the host ITS2 (Fig. 4.4) and 18S rRNA gene are shaded in grey. Values indicated on the branches represent  $>50\%$  bootstrap support (MP/ML/BI) and dashes denote  $<50\%$  bootstrap support. A global test of cospeciation determined an overall ParaFitGlobal = 0.011, prob = 0.0001 with 9,999 iterations using distance matrices derived from the maximum likelihood trees (Fig. 4.5 A & B). New nucleotide sequences are in bold.

#### **4.4.3 The cophylogenetic analysis of *Neoparamoeba* and their endosymbiotic PLO based on 18S rRNA gene sequences**

The PLO 18S rRNA gene alignment consisted of 1769 nucleotide sites and of these, 1507 (85%) were retained (Table 4.2) and 334 were parsimony-informative. The ML and BI analyses yielded four distinct lineages within the PLO group (rooted ML tree, clades A, B, C & D. Fig. 4.5B). Strains of PLOs derived from *N. pemaquidensis* (clade A, 94-97% support), *N. aestuarina* (clade B, 89-100%), *N. branchiphila* (clade C, 67%) and *N. perurans* (clade D, 100% support) clustered into individual monophyletic groups. The MP analysis of the PLO 18S rRNA gene sequence did not support the division of PLOs derived from *N. aestuarina* as a distinct monophyletic clade, but as a sister group to the PLO derived from *N. branchiphila* (data not shown). The truncated *Neoparamoeba* 18S rRNA gene alignment consisted of those strains from which the PLO 18S rRNA gene was sequenced and contained 2197 nucleotide sites, all of which were retained (Table 4.2) and 806 were parsimony-informative. Using the selected strains of *Neoparamoeba*, the ML and BI-derived phylogenies based on the *Neoparamoeba* 18S rRNA gene were consistent with the *Neoparamoeba* ITS2 phylogeny (Fig. 4.5A), while the MP analyses still retained *N. branchiphila* as the most divergent group (data not shown). Therefore the *Neoparamoeba* ML tree was used in the subsequent coevolutionary analyses. The cophylogenetic relationship between each *Neoparamoeba* strain and its endosymbiotic PLO was based on the genetic distances derived from the ML analyses described above. The global test indicated a highly significant cophylogenetic relationship (ParaFitGlobal = 0.011,  $p = 0.0001$  with 9,999



iterations). Using a significance threshold of  $p = 0.05$ , ParaFit analyses determined that all host-endosymbiont associations were significant ( $p < 0.05$ , data not shown).

#### 4.5 Discussion

In earlier studies, phylogenetic congruence was observed between PLOs and their host *Neoparamoeba* (Caraguel et al., 2007b; Dyková et al., 2008). This indicated that the endosymbiotic PLOs have cospeciated with their host *Neoparamoeba*. However, representatives of *N. perurans* were not assessed in previous coevolutionary analyses, thus the existence of a single ancient infection/colonisation that led to cospeciation between all PLOs and their host *Neoparamoeba* could not be corroborated. The initial objective of this study was to resolve phylogenetic ambiguities within the *Neoparamoeba* taxonomy. A pan-eukaryotic core of ITS2 secondary structure (Coleman, 2007; Schultz et al., 2005) was identified in strains of *N. pemaquidensis*, *N. branchiphila* and *N. aestuarina* and facilitated the alignment of their ITS2 nucleotide sequences. The ITS2-based phylogeny divided strains of *N. pemaquidensis* into two distinct clades, grouped strains of *N. aestuarina* within a separate monophyletic clade. These data were used to support the 18S rRNA gene-based cophylogenetic analyses that revealed strict phylogenetic congruence between all PLOs and their host *Neoparamoeba*.

Symbiotic associations are frequently observed phenomena that have a significant impact on the ecology and evolution of many eukaryotic and prokaryotic organisms

(Moran and Baumann, 2000; Moran et al., 2008; Zilber-Rosenberg and Rosenberg, 2008). Coevolutionary analysis of a symbiont and its host can be used to deduce the history of the symbiotic relationship. In the case of *Neoparamoeba* and their endosymbiotic PLOs, the strict monophyly of the endosymbiotic PLOs and an obligate symbiosis with their host (Dyková et al., 2003) imply that the mutualistic relationship has a single origin without secondary reversals to an independent state. In other words, PLOs appear to be vertically transmitted from one *Neoparamoeba* trophozoite host to its daughter cells. PLOs are probably transmitted during host mitotic division since *Neoparamoeba* always retain at least one intracellular PLO after clonal replication (Dyková et al., 2003; Dyková et al., 2000). The mechanisms that ensure PLOs are successfully transferred to the new host trophozoite remain enigmatic despite detailed ultrastructural morphological observations of PLOs and trophozoites in various stages of replication (Dyková et al., 2003). However, it does appear that transmission of PLOs is not the result of synchronised mitotic division since one to six PLOs can exist within a single *Neoparamoeba* host at any one time (Dyková et al., 2003; Dyková et al., 2008; Dyková et al., 2000; Dyková et al., 2005b; Dyková et al., 2007; Page, 1983; Young et al., 2007). Very little is known about the intimate relationship between PLOs and their host *Neoparamoeba* but the results of this study confirm that their association has been retained throughout the evolution of *Neoparamoeba* species.

The central question that has arisen from this study is whether the eukaryotic endosymbionts of *Janickina* and *Paramoeba*, that share morphological similarity to

PLOs (Hollande, 1980; Page, 1983; Page, 1987), represent one or multiple PLOs uptake events in separate species. Unfortunately, apart from one 18S rRNA gene sequence from a strain identified as *P. eilhardi* (AY686575) there are no phylogenetic data from *Paramoeba* and *Janickina* spp. (or their endosymbionts). Based on the 18S rRNA gene-based phylogeny *P. eilhardi* may represent an ancestral lineage of the *Neoparamoeba* however since no morphological characterisation of *P. eilhardi* was published (Peglar et al., 2003) its initial classification as a member of the *Paramoeba* is currently treated as preliminary without further characterisation. Thus detailed morphological and phylogenetic characterisation of representative strains of *Paramoeba* spp. and *Janickina* spp. (and their endosymbionts) is required to clarify whether the *Neoparamoeba*-PLOs relationship predates the separation of *Neoparamoeba* from their ancestral group.

The existence of an obligatory, physically intimate association between *Neoparamoeba* and their endosymbiotic PLOs (Dyková et al., 2003; Dyková et al., 2008; Dyková et al., 2005b) led to the classification of their relationship as a mutualistic symbiosis (Dyková et al., 2003; Dyková et al., 2008). However the benefits that either the endosymbiotic PLOs or host *Neoparamoeba* receive from this symbiotic relationship remain unclear. This study assessed the phylogenetic relatedness of the eukaryotic endosymbionts of *N. perurans* with PLOs from other *Neoparamoeba* species and pathogenic kinetoplastids of marine and freshwater fish (Grignard et al., 1996; Lom, 1979; Urawa, 1995; Urawa et al., 1991; Woo, 1994; Woo, 2003) on the premise that *N. perurans* trophozoites may be a “Trojan horse” and their endosymbionts were the true aetiological agents of AGD.

Since all PLOs and *Neoparamoeba* coevolved from a common ancestor, phylogenetic data provided no insight into the role of PLOs in AGD pathogenesis. Therefore, whilst it is still assumed that *Neoparamoeba* and PLOs receive some physical or nutritional benefits from their symbiotic relationship they are yet to be determined.

The ITS was assessed as an additional phylogenetic marker to assist in the taxonomic classification of *Neoparamoeba*. ITS1 and ITS2 nucleotide sequences are removed via splicing during the process of transcription and therefore thought to be subject to mild functional constraints leading to nucleotide and length variation (Álvarez and Wendel, 2003). Whilst ITS1 and ITS2 display a high degree of nucleotide sequence variability, their secondary structure is conserved due to their structural role in pre-RNA processing (Álvarez and Wendel, 2003; Good et al., 1997; Michot et al., 1999). Therefore, utilising the high mutation rate in ITS1 and ITS2 nucleotide sequences for phylogenetic analyses requires consideration of both sequence and structure when calculating the nucleotide sequence alignment (Coleman, 2003; Coleman, 2007; Schultz et al., 2005; Seibel et al., 2006; Wolf et al., 2005). A conserved secondary structure was only identified for the ITS2 from strains of *N. pemaquidensis*, *N. branchiphila* and *N. aestuarina* and was similar to the common core of the ITS2 secondary structure observed across the Eukaryota (Coleman, 2007; Schultz et al., 2005). Failure to predict the secondary structure of ITS1 and ITS2 from *Neoparamoeba* species does not necessarily mean they lack a conserved secondary structure, it may only reflect the lack of information that is currently available for these gene regions. For instance, homology-based helix and

structural modelling identified less than half of 50,000 available ITS2 secondary structures from eukaryotes (Wolf et al., 2005). Further understanding of the structural role of ITS in transcription (Good et al., 1997; Michot et al., 1999), additional sequencing and predictive modelling will facilitate the identification of unknown ITS1 and ITS2 secondary structures expanding the usefulness of ITS in phylogenetic studies.

Analysis of the *Neoparamoeba* ITS2 indicated that there are two genetically distinct populations within strains of *N. pemaquidensis*. However, other than the ITS2-based phylogenetic tree topology there was no additional evidence based on their morphology, geographic origin or host preference that would be useful for defining subclades of *N. pemaquidensis*, as observed in previous studies using the 18S rRNA gene-based phylogeny (Dyková et al., 2005b; Dyková et al., 2007). Furthermore, one strain of *N. pemaquidensis* used in this study, CCAP1560/4, did not cluster into either of the subclades. Therefore, without additional phenotypic support to justify the division of *N. pemaquidensis* into subclades these data should be treated as preliminary.

The results presented here support and extend previous observations of intragenomic variability within the ITS2 of *Neoparamoeba* (Caraguel et al., 2007b). Such variation within samples is indicative of the presence of multiple alleles within a single individual. Eukaryotes usually possess multiple copies of the ITS2, as an element of the rRNA gene family, present in tandem arrays clustered within the genome (Hillis and Dixon, 1991). In eukaryotes, multi-gene rRNA families can encounter three evolutionary fates, either

all repeat types are maintained, new repeat types are generated or repeat types are lost through a process of concerted evolution (Álvarez and Wendel, 2003; Brown et al., 1972). In most eukaryotes the ITS retains a high degree of homology between alleles through molecular turnover mechanisms, such as concerted evolution, making them the ideal candidate for phylogenetic analyses (Coleman, 2003; Hillis and Dixon, 1991). Despite observing significant intragenomic heterogeneity within the ITS2 of *Neoparamoeba* it remained phylogenetically informative and displayed a high degree of congruence with the phylogeny inferred from the 18S rRNA gene. Thus, within the ITS2 some level of concerted evolution (i.e. nucleotide sequence homogenisation) is presumed to have occurred at a rate faster than speciation. Furthermore, high levels of intragenomic variability in the ITS2 did not interfere with the common core of predicted secondary structure amongst *N. pemaquidensis*, *N. branchiphila* and *N. aestuarina* indicating that the ITS2 would retain its functional role in the pre-rRNA processing (Álvarez and Wendel, 2003; Good et al., 1997; Michot et al., 1999). It is uncertain why *Neoparamoeba* retain a higher degree of ITS2 intragenomic heterogeneity than other amoebic species (De Jonckheere, 2004; Som et al., 2000; Stothard et al., 1998) although variable rates of concerted evolution have also been observed in the ITS of individuals from a variety of taxa (for example Bart et al., 2008; Bezzhonova and Goryacheva, 2008; Schlotterer et al., 1994; Tang et al., 1996; Wörheide et al., 2004). It must also be acknowledged that the intragenomic heterogeneity observed in this study may be in part due to PCR sequencing error (Kobayashi et al., 1999). Using similar PCR sequencing techniques Dyková (2005b) reported low intragenomic heterogeneity (0.73 to 2.36%)

between 18S rRNA gene alleles in PCR products obtained from three *Neoparamoeba* clones. Therefore, whilst sequencing error may account for a minor portion of the variability there is little evidence to suggest that it could account for the levels of intragenomic variability observed in this study. Assuming that nucleotide sequence heterogeneity is attributable to polymorphism within the ITS2 repeats, knowledge of the occurrence of paralogues and the degree of intragenomic variability should be an important basis for evolutionary interpretations in phylogenetic studies using *Neoparamoeba* ITS2 alone.

Based on the data presented here, strict phylogenetic congruence between PLOs and their host *Neoparamoeba* was demonstrated implying that their relationship is strictly hereditary and most likely the result of direct vertical transmission of PLOs from host to host. Therefore, molecular and morphological characterisation of *Janickina*, *Paramoeba* and their PLO-like endosymbionts will reveal whether their coevolutionary relationships share common ancestry with PLOs and their host *Neoparamoeba*. Further studies are required in order to understand what benefits that *Neoparamoeba* and PLOs receive from their mutualistic symbiosis. In order to do this, studies could make use of comparative genomics due to the technical difficulties of examining an obligate symbiotic relationship where neither the endosymbiotic PLOs, nor the *Neoparamoeba* host are independently culturable (Dyková et al., 2003; Dyková et al., 2000).

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**5    DEVELOPMENT OF A DIAGNOSTIC PCR TO DETECT**  
***NEOPARAMOEBA PERURANS*, AGENT OF AMOEBIC GILL DISEASE**

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Published in:

Young N. D., Dyková I., Nowak B. F. and Morrison R. N. (2008) Development of a diagnostic PCR to detect *Neoparamoeba perurans*, agent of amoebic gill disease (AGD). *Journal of Fish Diseases* **29**, 1-11.



## 5.1 Abstract

The recent description of *Neoparamoeba perurans* as an aetiological agent of amoebic gill disease (AGD) advanced our understanding of the condition and has forced a re-evaluation of methods used for the diagnosis of AGD. Currently, there are no tools available that are both specific for *N. perurans* and suitable for a routine diagnostic procedure. Therefore, in this study we describe an assay to detect *N. perurans*. The assay, which utilizes PCR to amplify the *N. perurans* 18S rRNA gene, was shown to be specific and highly sensitive. *N. perurans* was detected in both gill samples and primary isolates of non-cultured gill-derived amoebae obtained during necropsy or biopsy from AGD-affected Atlantic salmon, *Salmo salar*. The PCR-based assay provides a simple, flexible tool that will be a useful addition to the diagnostic repertoire for AGD. It may also be used for the genotypic screening of trophozoites during culture and could facilitate further epidemiological and ecological studies of AGD.

## 5.2 Introduction

Amoebic gill disease (AGD) is an ectoparasitic condition of some farm-reared marine fish (Dyková et al., 1995; Kent et al., 1988; Munday, 1986). AGD is characterised by multifocal lesions that appear as pale gill tissue (Munday et al., 1990; Rodger and McArdle, 1996). In Tasmania, Australia, the prevalence and density of these gross gill lesions is monitored by commercial marine Atlantic salmon (*Salmo salar*) growers in order to estimate the severity of AGD and manage treatment (Adams et al., 2004; Clark and Nowak, 1999; Clark et al., 2003). Other agents can elicit gill lesions macroscopically indistinguishable from AGD-related lesions (Adams et al., 2004; Clark et al., 1997) and all farm-based assessments of AGD are therefore presumptive. In the laboratory, histological examination of gill tissues has been a reliable method for diagnosing AGD (Dyková and Novoa, 2001). Typically, the final diagnosis is confirmed when trophozoites that possess one or more endosymbiotic *Perkinsela amoeba*-like organisms (PLOs) (Dyková et al., 2003) are detected in close association with hyperplastic gill lesions (Dyková and Novoa, 2001). However, using histological examination, there is limited capacity to characterise the infectious agent and diagnoses are again presumptuous.

For many years *Neoparamoeba pemaquidensis* was considered the aetiological agent of AGD. This conclusion was based on morphological (Kent et al., 1988) and molecular (Wong et al., 2004) characterisation of trophozoites, particularly those cultured from the gills of fish affected by AGD. Several techniques such as PCR (Wong et al., 2004) were

developed to detect *N. pemaquidensis*. Immunohistochemical, immuno-fluorescent antibody test (IFAT) or immuno-dot blot detection of trophozoites using polyclonal antisera raised against *N. pemaquidensis* were all used to confirm AGD in gill tissues presumptively diagnosed by histological examination (Douglas-Helders et al., 2001a; Howard, 2001; Nowak et al., 2002). However, the isolation of *Neoparamoeba branchiphila* from AGD-affected fish meant that AGD may be a disease of mixed aetiology and that *N. pemaquidensis*-specific diagnostics were of limited use. Subsequently, an additional diagnostic PCR for the detection of *N. branchiphila* was developed (Dyková et al., 2005b).

The most recent addition to the *Neoparamoeba* Page, 1987 genus was *Neoparamoeba perurans*. Young (2007) developed *in situ* hybridisation (ISH) probes to detect *N. perurans*, *N. pemaquidensis* and *N. branchiphila* yet interestingly, *N. perurans* was the only detectable species in AGD-affected gill tissue from Atlantic salmon cultured in South-East Tasmania, Australia. In fact *N. perurans* was the only detectable species in four species of fish cultured across six countries (Young et al., 2008c). While ISH assays complement histological examination, the technique is expensive, time consuming and only suitable for fixed gill tissue samples. Therefore, given the emergence of *N. perurans* as an aetiological agent of AGD, we aimed to develop a simpler and more flexible detection assay that could be utilised in both field and laboratory investigations. Oligonucleotides that amplify the *N. perurans* 18S rRNA gene were designed and the PCR amplification specificity was validated empirically. The new PCR assay is both

specific and sensitive and we show that the assay can be used to detect *N. perurans* in primary amoebae isolates and clinical samples from AGD-affected Atlantic salmon.

### **5.3 Materials and methods**

#### **5.3.1 Acquisition of clonal, cultured and non-cultured gill-derived (NCGD) amoebae**

AGD-affected Atlantic salmon, *S. salar* were obtained from a tank-based population of fish infected by cohabitation and maintained at the School of Aquaculture, University of Tasmania (Launceston, Tasmania, Australia). Fish were anaesthetised (50 mg L<sup>-1</sup> Aqui-S NZ Ltd, Lower Hutt, New Zealand) and assessed for gross AGD-like lesions as previously described (Munday et al., 2001). Fish presumptively diagnosed with AGD were euthanized (100 mg L<sup>-1</sup> Aqui-S) and amoebae were isolated from gill tissues as previously described (Morrison et al., 2004), herein termed NCGD amoebae.

Clonal cultures of amoeba strains were obtained from a culture collection held at the School of Aquaculture, University of Tasmania (Table 5.1). Identification of these strains was based on phylogeny inferred from their 18S rRNA gene sequences (Dyková et al., 2005b; Dyková et al., 2007; Fiala and Dyková, 2003; Peglar et al., 2003; Wong et al., 2004; Young et al., 2007). Amoebae culture and harvesting procedures followed those previously described (Dyková et al., 2000; Dyková et al., 2005b). Aliquots of each amoeba strain containing between  $0.7\text{--}7.2 \times 10^5$  trophozoites were centrifuged (10,000 × g, 1 min), the supernatant removed and the cell pellets stored at -80°C.

### **5.3.2 Acquisition of samples from AGD-affected Atlantic salmon**

AGD-affected Atlantic salmon were obtained from a tank-based population of fish infected by cohabitation and maintained at the School of Aquaculture, University of Tasmania (Launceston, Tasmania, Australia). Fish were checked regularly for signs of morbidity. Six moribund fish presumptively diagnosed with AGD were euthanized (100 mg L<sup>-1</sup> Aquai-S). The surfaces of the anterior and posterior hemibranchs on the right-hand side of the fish were gently wiped using a sterile cotton-tip swab. The swab was then immersed in 500 µL sterile PBS and stored at -20°C (gill swab PBS). This process was repeated with the hemibranchs of the left-hand side of the fish using a new cotton-tip swab which was subsequently stored dry at room temperature (gill swab dry). A section of gill tissue with an AGD-like gill lesion was removed, immersed in 100% ethanol and stored at -20°C. The remaining gill tissue was excised and placed into seawater Davidson's fixative for 24 h then stored in 70% ethanol at room temperature. The second left, anterior hemibranch was processed, sectioned (5 µm) and stained with H&E following routine histological procedures. AGD gill lesions were identified by histological examination using established criteria (Dyková and Novoa, 2001). The severity of AGD was expressed as the proportion of gill filaments exhibiting AGD lesions in each section (Adams and Nowak, 2004b). A filament was counted only when the central venous sinus was visible in at least two-thirds of the filament. When incidental AGD-related mortalities occurred, post-mortem sampling was carried out on six fish as described above during necropsy. However, due to extensive post-mortem autolysis, these gill tissues were not examined histologically. Negative control biopsy

samples were also taken from six AGD-naïve Atlantic salmon obtained from a tank-based population of fish maintained at the School of Aquaculture, University of Tasmania (Launceston, Tasmania, Australia) as described above.

### **5.3.3 DNA extraction**

Genomic DNA was extracted from trophozoites and gill tissues using a DNeasy Tissue Kit (Qiagen, Doncaster, Victoria, Australia) as per the manufacturer's instructions.

Genomic DNA was extracted from cotton-tipped swabs using a DNeasy Tissue Kit (Qiagen) with minor modifications to the manufacturer's instructions. Initially, gill swab PBS samples were defrosted then both the gill swab PBS and gill swab dry samples were transferred to individual 2.0 mL centrifuge tubes. ATL buffer (180 µL, Qiagen) and proteinase K (20 µL, Qiagen) were added, the tubes were vortexed briefly and incubated at 56°C overnight. The liquid was carefully recovered by pressing the cotton-tip of the swab against the inside of the centrifuge tube. The swabs were discarded and the remaining procedures were as per the manufacturer's instructions. The total DNA concentration was determined for each sample using a fluorochrome bis-benzimidazole assay (Hoechst 33258; DNA Quantitation Kit, BioRad, Regents Park, New South Wales, Australia) as per manufacturer's instructions.

### **5.3.4 18S rRNA gene PCR amplification with *N. perurans* oligonucleotides**

Consensus 18S rRNA gene sequences were generated using GenBank

(<http://www.ncbi.nlm.nih.gov/>) sequence entries for *N. perurans* (GenBank accession

numbers EF216899 to EF216905), *N. pemaquidensis* (AF371968 to AF371970, AY183887, AY183889, AY193722, AY193723, AY686577, AY686578, AY714350 to AY714363), *Neoparamoeba aestuarina* (Page, 1970) (AY121848, AY121851, AY121852, AY686574), *N. branchiphila* (AY193724 to AY193726, AY714365 to AY714367) and Atlantic salmon (AJ427629). The consensus sequences were aligned in ClustalX (Thompson et al., 1997) (gap opening/gap extension penalty = 8/2). PCR oligonucleotides were designed in Primer Premier 5 (Premier Biosoft International, Palo Alto, California, USA) to anneal to the *N. perurans* consensus sequence at loci that were unambiguously divergent from the 18S rRNA gene sequences of other *Neoparamoeba* species and Atlantic salmon. Oligonucleotides were designed (F-5'-ATCTTGACYGGTTCTTTTCGRGA-3' and R-5'-ATAGGTCTGCTTATCACTYATTCT-3') and then assessed for sequence identity with other species using BLASTn analysis (NCBI, <http://www.ncbi.nlm.nih.gov/>). The ability to PCR amplify DNA templates from both clonal culture and NCGD amoebae was assessed using universal 18S rRNA gene oligonucleotides [18e and 18i (Hillis and Dixon, 1991)] as previously described (Mullen et al., 2005). Amplification of the 18S rRNA gene with *N. perurans* or universal 18S rRNA gene oligonucleotides was performed in volumes of 25 µL containing 20 ng DNA (unless specified), 0.625 U of BIOTAQ DNA Polymerase (Bioline, Alexandria, New South Wales, Australia), 1× NH<sub>4</sub> BIOTAQ reaction buffer (Bioline), 250 µM of each deoxynucleotide triphosphate (dNTP, dATP, dCTP, dGTP, and dTTP), 1.5 mM of MgCl<sub>2</sub> and 0.4 µM of each oligonucleotide. PCR cycle conditions whilst using the universal 18S rRNA gene

oligonucleotides were 94°C for 3 min; 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, for 35 cycles; and 72°C for 10 min. PCR cycle conditions whilst using the *N. perurans* oligonucleotides were 94°C for 3 min; 94°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec, for 35 cycles; and 72°C for 10 min. Genomic DNA from Atlantic salmon was used as a host template control, whereas reactions containing either no template or plasmid DNA housing the full-length 18S rRNA gene of *N. perurans* (GenBank accession number EF216901) were used as negative and positive controls respectively. PCR reactions were electrophoresed through 1 to 2% agarose/tris-borate EDTA buffer and visualized by staining with 1 µg/ml ethidium bromide. The specificity of *N. perurans* oligonucleotides was confirmed by sequencing a PCR-amplified product. The methods used to ligate the PCR product into a plasmid vector, transformation of *E. coli*, colony selection and plasmid purification were as previously described (Young et al., 2007). Sequencing reactions were initiated using either the *N. perurans* forward or reverse oligonucleotide and analysed on an ABI 3730xl DNA analyser (Applied Biosystems (ABI), Scoresby, Victoria, Australia).

## **5.4 Results**

### **5.4.1 Specificity of *N. perurans* oligonucleotides using sequence alignment and PCR.**

Oligonucleotides were designed to specifically PCR amplify a 636 bp region of the *N. perurans* 18S rRNA gene (Fig. 5.1A). The specificity of *N. perurans* oligonucleotides was then assessed empirically using genomic DNA from 15 strains of *N. pemaquidensis*,



one strain of *N. aestuarina*, four strains of *N. branchiphila*, one strain of *Paraflabellula hoguae*, 11 primary isolates of NCGD amoebae from AGD-affected Atlantic salmon and Atlantic salmon as well as purified plasmid DNA containing the entire 18S rRNA gene from *N. perurans*. The *N. perurans* oligonucleotides primed the purified plasmid containing the *N. perurans* 18S rRNA gene producing a PCR product of the expected size (Fig. 5.1B & C, upper rows). PCR products were consistently amplified using genomic DNA from NCGD amoebae (Fig. 5.1C, upper row) yet no PCR products were amplified using template DNA from the other *Neoparamoeba* species, *P. hoguae* or Atlantic salmon (Fig. 5.1B & C upper rows). Furthermore the DNA sequences from amplicons were >99% homologous with the 18S rRNA gene of *N. perurans*.

To illustrate that there was an equal amount of template in each reaction and that all DNA templates were amplifiable by PCR, control PCR reactions containing universal 18S rRNA gene oligonucleotides were performed. Indeed, the universal 18S rRNA gene oligonucleotides yielded PCR products of the expected size (approximately 440 bp) across all templates tested (Fig. 5.1B & C, lower rows). No PCR products were amplified with either oligonucleotide pair in control reactions that did not contain template DNA (Fig. 5.1B & C, upper & lower rows).

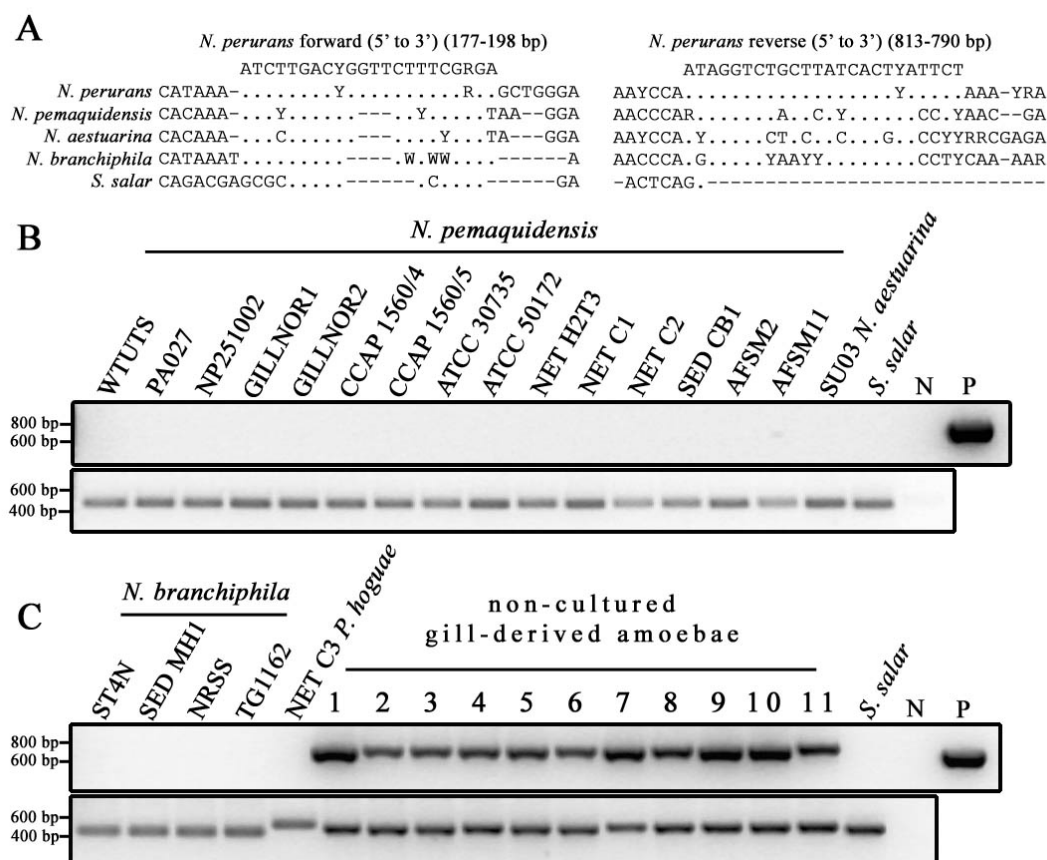


Fig. 5.1. Specificity of oligonucleotides designed to PCR amplify a 636 bp region of the *Neoparamoeba perurans* 18S rRNA gene. (A) Alignment of *N. perurans* oligonucleotides against consensus 18S rRNA gene sequences of known *Neoparamoeba* species and Atlantic salmon (*Salmo salar*). The 18S rRNA gene sequences aligned with the antisense oligonucleotide were reverse complemented for presentation. Mixed bases are shown using mixed base nomenclature standards (NC-IUB, 1985). Consensus in bases is represented in the alignment as a dot (.) while a gap in the sequence is represented by a dash (-). PCR amplification using the *N. perurans* oligonucleotides (Fig 5.1B and Fig 5.1C upper) and universal 18S rRNA gene oligonucleotides (Fig. 5.1B and Fig. 5.1C lower) with 20 ng of genomic DNA from *Neoparamoeba pemaquidensis*, *Neoparamoeba branchiphila*, *Neoparamoeba aestuarina*, *Paraflabellula hoguae* and non-cultured gill-derived (NCGD) amoebae. Control PCR reactions included genomic DNA from Atlantic salmon, no DNA template control (N) and a positive control (P) consisting of purified plasmid DNA containing the entire 18S rRNA gene of *N. perurans* (GenBank accession number EF216901). PCR reactions presented as Fig. 5.1B upper and Fig. 5.1C upper or Fig. 5.1B lower and Fig. 5.1C lower were run simultaneously and analysed by agarose gel electrophoresis separately.

#### **5.4.2 Sensitivity of PCR using *N. perurans* oligonucleotides**

The sensitivity of PCR using *N. perurans* oligonucleotides was assessed using template DNA from NCGD amoebae (Fig. 5.1C, primary isolates 7, 8 and 10). PCR products were amplified using  $\geq 0.2$  pg of NCGD amoebae template DNA, equivalent to the detection of  $\geq 0.05$  amoeba (Fig. 5.2A, B & C, upper rows). The addition of 20 ng of Atlantic salmon genomic DNA to each reaction reduced the PCR amplification efficiency with PCR products only detectable in reactions with  $\geq 2$  pg of NCGD amoebae template DNA, equivalent to  $\geq 0.5$  amoeba per reaction (Fig. 5.2A, B & C, lower rows). The number of amoebae per PCR reaction are estimates given that there was variability in the DNA extraction efficiency from NCGD amoebae primary isolates [20 ng DNA per  $6283 \pm 1527$  amoebae (mean  $\pm$  standard error of the mean,  $n = 11$ )].

#### **5.4.3 Pathology of AGD gill lesions sampled at necropsy and biopsy**

Macroscopic gill lesions were observed along the gill filaments in all AGD-affected Atlantic salmon and presented as focal patches of pale gill tissue, typical of AGD-like lesions. These lesions were observed in all AGD-affected Atlantic salmon during necropsy (Fig. 5.3A) or less prominently on the gills of AGD-affected Atlantic salmon prior to taking gill biopsies (Fig. 5.3B).

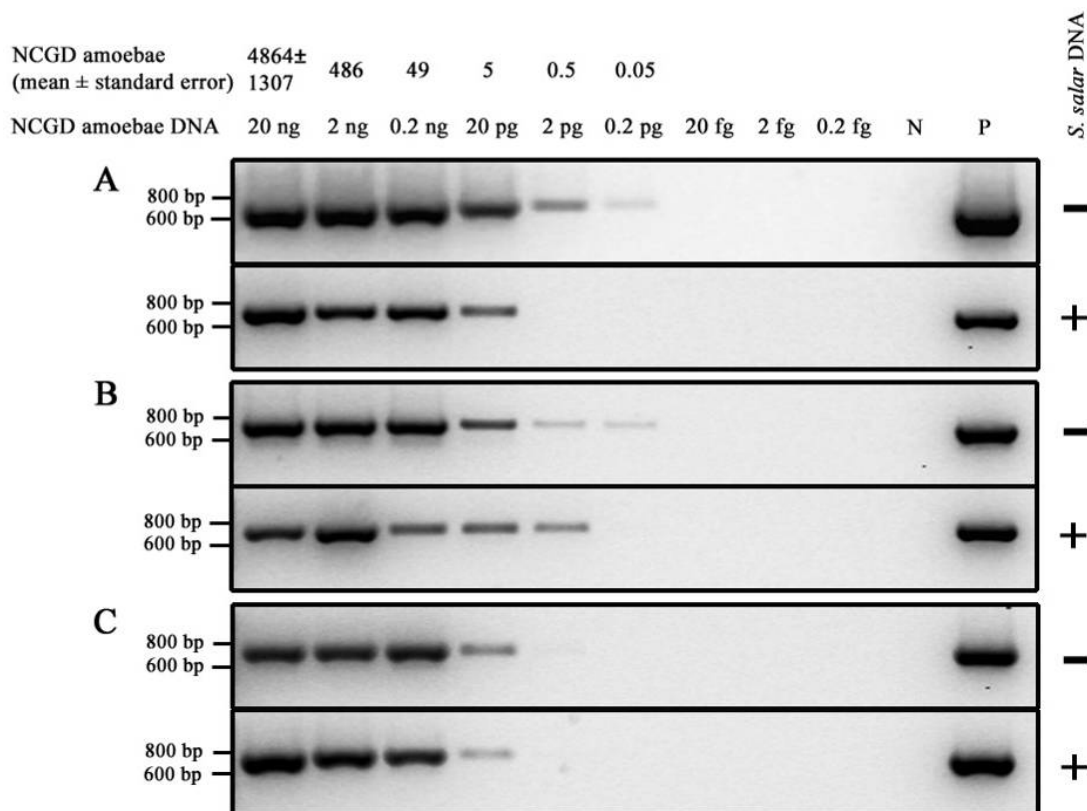


Figure 5.2. The sensitivity of PCR using *Neoparamoeba perurans* oligonucleotides. Amplification of a 636 bp region of the 18S rRNA gene of primary isolates 7 (A), 8 (B) and 10 (C) of non-cultured gill-derived (NCGD) amoebae from AGD-affected Atlantic salmon (*Salmo salar*) shown in Fig. 5.1C. Genomic DNA was diluted 10-fold to obtain 20 ng to 0.2 fg of template DNA in the absence (*S. salar* DNA -) or presence (*S. salar* DNA +) of 20 ng of Atlantic salmon genomic DNA per reaction. Control PCR reactions included no DNA template control (N) and a positive control (P) consisting of purified plasmid DNA containing the entire 18S rRNA gene of *N. perurans* (GenBank accession number EF216901). The mean number of NCGD amoebae per reaction is estimated from the number of amoebae per 20 ng of DNA of the three primary isolates of NCGD amoebae used.

A final diagnosis of AGD was confirmed in five of six gill biopsies from AGD-affected Atlantic salmon by histological examination. The structural changes observed in sections were consistent with the pathology described in other cases of AGD (Dyková et al., 1995; Kent et al., 1988; Munday et al., 1990; Rodger and McArdle, 1996; Roubal et al.,

1989). The most prominent feature was the extensive fusion of secondary lamellae due to the hyperplasia of epithelial-like tissue (Fig. 5.3C). Amoebae were located at the distal ends of fused lamellae (Fig. 5.3C) and contained one or more perinuclear eosinophilic bodies, representative of the endosymbiotic *Perkinsiella amoebae*-like organism (PLO) (Dyková et al., 2003) (Fig. 5.3C insert). The severity of AGD in gill biopsies was reported as the proportion of AGD-affected gill filaments in each section examined histologically (Fig. 5.4). No pathological changes were observed in AGD-naïve Atlantic salmon (data not shown).

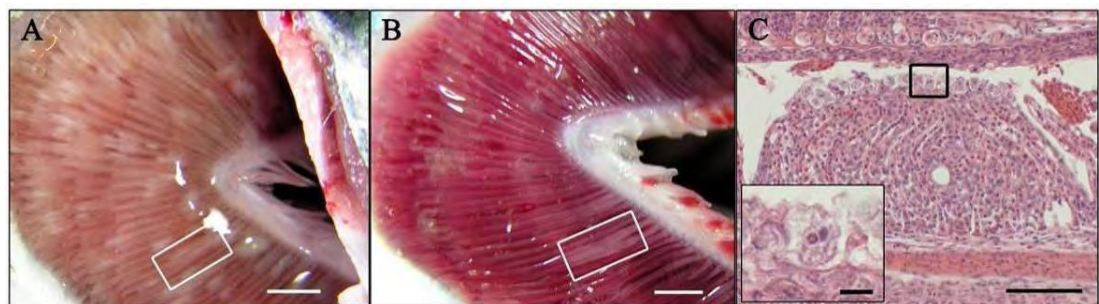


Fig. 5.3. Pathology of Atlantic salmon (*Salmo salar*) presumptively diagnosed with amoebic gill disease (AGD). Figs. 5.3A & B represent gross pathology of hemibranchs with lesions presumed to be AGD-related. The boxed areas on Figs. 5.3A & B represent the gill tissue removed during necropsy (A) or as gill tissue biopsies (B). Fig. 5.3C represents a typical AGD-gill lesion observed in gill biopsies. Associated with changes in the gill tissue structure were amoebae containing endosymbiotic *Perkinsiella amoebae*-like organisms (magnified insert of outlined box). H&E stained, 5 µM section. Scale bars represent 3 mm (A & B), 100 µm (C) and 10 µm (C insert).

#### **5.4.4 Detection of *N. perurans* in gill samples from AGD-affected Atlantic salmon taken during necropsy or biopsy by PCR**

Employing the *N. perurans*-specific oligonucleotides, PCR amplification products, whose size was consistent with the expected amplicon, were obtained from gill samples of AGD-affected Atlantic salmon taken during necropsy or biopsy (Fig. 5.4). No PCR products were amplified from gill samples taken from AGD-naïve Atlantic salmon (data not shown). Reactions that failed to amplify a PCR product were further assessed by replicating the PCR reaction using 100 ng of DNA template, resulting in no additional amplification of PCR products (data not shown). PCR products were amplified in all gill swab PBS samples from AGD-affected Atlantic salmon (Figure 5.4, fish 1-12). Only one of six gill swab dry samples taken during biopsy of AGD-affected Atlantic salmon amplified PCR products (Fig. 5.4 fish 1). However, PCR products were amplified in all six gill swab dry samples taken during necropsy (Fig. 5.4 fish 7-12). PCR products were amplified in five of the six gill tissue biopsies from AGD-affected Atlantic salmon (Fig. 5.4, fish 1, 2, 3, 4 and 6) and all gill tissues taken during necropsy (Fig. 5.4, fish 7-12). In most cases, the PCR products amplified from gill samples taken during necropsy had qualitatively higher DNA yields than PCR products amplified from gill samples taken during biopsy.

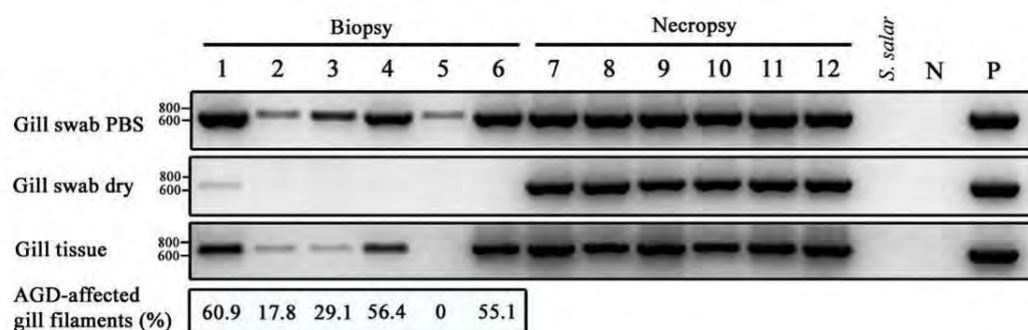


Fig. 5.4. Successful PCR amplification of a 636 bp region of the 18S rRNA gene using *Neoparamoeba perurans* oligonucleotides and genomic DNA from gill samples removed during necropsy or biopsy. Biopsy samples from the gills of six replicate fish presumptively diagnosed with AGD based on gross pathology and subsequently confirmed using histological examination. Gill samples taken from six replicate fish during necropsy presumptively diagnosed with AGD based on gross pathology. PCR amplification of template DNA extracted from gill swabs stored in PBS at -20°C, gill swabs stored dry at room temperature or gill tissue from AGD-affected fish. The severity of AGD was determined during histological examination and is reported as the proportion of gill filaments affected by AGD. Control PCR reactions included genomic DNA from Atlantic salmon (*Salmo salar*), no DNA template control (N) and a positive control (P) consisting of purified plasmid DNA containing the entire 18S rRNA gene of *N. perurans* (GenBank accession number EF216901).

## 5.5 Discussion

Numerous laboratory tools have been developed to detect *N. pemaquidensis* or *N. branchiphila* and in some instances, these have been used as AGD diagnostics (Douglas-Helders et al., 2001a; Howard, 2001; Nowak et al., 2002). However, the value of these tools as diagnostics was undermined when recently, it was shown that *N. perurans* but not *N. pemaquidensis* nor *N. branchiphila* is consistently associated with AGD lesions (Young et al., 2007; Young et al., 2008c). Other methods, such as histological examination of gill tissue, are reliable for diagnosing AGD (Dyková and Novoa, 2001) but are constrained by an inability to identify the infectious agent. By histological

examination alone, the agent can only be restricted to three genera, *Paramoeba*, *Neoparamoeba* or *Janickina* on account of the presence of a perinuclear PLO in gill lesion-associated trophozoites (Dyková et al., 2000).

In this study we describe a PCR-based assay to detect *N. perurans* using oligonucleotides designed to anneal with regions of the 18S rRNA gene. This gene was chosen for the advantages in assay (oligonucleotide) design and the availability of gene sequences. In addition, multiple copies of the 18S rRNA gene are encoded within the eukaryotic genome (Long and Dawid, 1980) enhancing the sensitivity of PCRs. A significant 18S rRNA gene database has developed and currently includes 48 sequences from the genus *Neoparamoeba*, mostly for the purpose of molecular phylogenetic analyses. Indeed, the taxonomy of *Neoparamoeba* species is largely derived from phylogenetic inference using these 18S rRNA gene sequences (Dyková et al., 2005b; Dyková et al., 2007; Fiala and Dyková, 2003; Mullen et al., 2005; Peglar et al., 2003; Young et al., 2007). In terms of oligonucleotide design, the 18S rRNA gene offers both conserved and variable regions (Hillis and Dixon, 1991). Alignment of 18S rRNA gene sequences from closely-related species reveals variable regions that can be used to design species-specific oligonucleotides.



Nucleic acid amplification techniques are increasingly relevant for the diagnosis of infectious diseases caused by protozoan parasites (Weiss, 1995; Yera et al., 2003). PCR-based assays provide advantages over other methods used for the routine diagnosis of parasitic infection, particularly when parasitaemia is low and differentiation between morphologically identical species is required (Weiss, 1995). For example, PCR-based techniques are useful to rapidly differentiate between the pathogenic amoeba, *Entamoeba histolytica* and the microscopically identical, but non-pathogenic *Entamoeba dispar* (DiMiceli, 2004; Evangelopoulos et al., 2000; Freitas et al., 2004; Gonin and Trudel, 2003; Kebede et al., 2004; Lebbad and Svard, 2005; Sanuki et al., 1997). The discrimination between morphologically identical species is of particular relevance to the current study given that, at present, all four members of the *Neoparamoeba* genus are believed to be morphologically indistinguishable (Dyková et al., 2000; Dyková et al., 2005b; Young et al., 2007) yet only *N. perurans* has been confirmed as an agent of AGD (Young et al., 2007; Young et al., 2008c). Of the 32 amoebae isolates tested here, the *N. perurans* PCR effectively amplified genomic DNA from *N. perurans* but not *N. pemaquidensis*, *N. branchiphila* nor *N. aestuarina*.

The principal advantage of PCR-based assays is that they are highly sensitive, whilst simultaneously maintaining specificity (Tang et al., 1997; Weiss, 1995; Yera et al., 2003). The sensitivity of a PCR assay determines the range in which the template DNA can be detected, thus indicating the likelihood of reporting a false-negative result

(template present, but not amplified). The PCR method used in this study is highly sensitive, as evidenced by the detection of template from less than one trophozoite. Seeding genomic DNA of NCGD amoebae isolates with genomic DNA from Atlantic salmon reduced the PCR sensitivity approximately 10-fold although the assay remained sensitive to less than one amoeba. This reduction in PCR efficiency is typical in the presence of non-target DNA or other contaminants (see review Wilson, 1997). Whilst seeding *N. perurans* DNA template with host DNA is not equivalent to detecting *N. perurans* in host tissue naturally colonised by *N. perurans*, this technique does demonstrate that the *N. perurans* oligonucleotides reliably amplify PCR products within mixed DNA templates and provides a useful starting point for the optimisation of experimental procedures. The requirement for 5 amoebae or 20 pg of DNA extracted from amoebae is consistent with other amoeba-specific PCR assays (Hamzah et al., 2006; Khan et al., 2001; Myjak et al., 2000).

*N. perurans* DNA was consistently amplified from all fish using all three sampling methods during necropsy. Whilst this may occur due to a post-mortem autolytic change in the ratio of host genomic DNA to *N. perurans* genomic DNA it may also suggest that *N. perurans* proliferate on the gills of fish post-mortem, consistent with previous observations that trophozoites can survive (Dyková and Novoa, 2001) and proliferate (Douglas-Helders et al., 2000) on the gills of dead Atlantic salmon. However, it is not possible to eliminate post-mortem colonisation of gill tissue and this will be problematic

in future, when retrospectively demonstrating the presence of *N. perurans* in gill tissues during necropsy. Unlike samples obtained during necropsy, there was variable amplification of DNA from biopsy samples. Only the gill swab dry sample with the highest severity of AGD (60.9%) amplified suggesting that this sampling technique is not suitable or requires optimisation. The DNA from five of six gill tissue biopsy samples amplified and these data were consistent with histological examination. In cases of AGD, the number of amoebae obtained during sampling could be low due to the gregarious distribution of amoebae (Adams and Nowak, 2004b; Munday et al., 1990) or the reduction in the number of amoebae observed on AGD gill lesions over the latter stages of pathogenesis (Adams and Nowak, 2003; Dyková et al., 1995). Under these circumstances, the PCR-based assay would be useful in confirming the presence of *N. perurans* in AGD-affected fish. Indeed, in one instance described here, *N. perurans* was detected from a gill swab PBS sample taken from a fish that did not present AGD lesions during gross or histological examination. Nor did the DNA from the gill tissue biopsy amplify by PCR. This suggests that the PCR may provide superior sensitivity over methods traditionally used to diagnose AGD.

In this study, the PCR assay was applied to gill samples from AGD-affected Atlantic salmon and primary isolates of NCGD amoebae. However, the PCR assay could easily be adapted for the detection of *N. perurans* from different biological samples. The prevalence of human diseases directly or indirectly attributable to amoebae have been

monitored in environmental samples using PCR-based assays (Kilvington and Beeching, 1995; Kuiper, 2006; Kuiper et al., 2006; Pelandakis and Pernin, 2002). *N. perurans* has now been demonstrated to be a cosmopolitan aetiological agent of AGD in some temperate marine fish world-wide (Young et al., 2008c). However, there are few data available on this recently discovered species and therefore the PCR would be valuable for epidemiological and ecological studies of AGD.

In summary, we have developed a new specific and sensitive PCR assay for the detection of *N. perurans*. In conjunction with a commercial DNA extraction kit, the technique is rapid, relatively safe and easily employed in both lethal and non-lethal field and laboratory sampling. The PCR assay reliably detected *N. perurans* from gill swabs and gill tissue from AGD-affected Atlantic salmon and these data provide a platform for adaptation of the assay into research projects and routine diagnostic screening programs for the aquaculture industry.

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**6 COORDINATED DOWN-REGULATION OF THE ANTIGEN  
PROCESSING MACHINERY IN THE GILLS OF AMOEBIC GILL  
DISEASE-AFFECTED ATLANTIC SALMON (*SALMO SALAR*)**

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## 6.1 Abstract

Several important cultured marine fish are highly susceptible to an ectoparasitic condition known as amoebic gill disease (AGD). In AGD-affected fish, modulation of IL-1 $\beta$ , p53 and p53-regulated transcripts is restricted to the (multi)focal AGD-associated gill lesions. To determine whether this lesion-restricted modulation of transcripts occurs on a transcriptome-wide scale and to identify mechanisms that underpin the susceptibility of fish to AGD, we compared the transcriptome of AGD lesions with “normal” tissue from AGD-affected and healthy individuals. Global gene expression profiling using a 16K salmonid microarray, revealed a total of 176 significantly regulated annotated features and of those, the modulation of 99 (56%) was lesion-restricted. Annotated transcripts were classified according to functional gene ontology. Within the immune response category, transcripts were almost universally down-regulated. In AGD-affected tissue significant, coordinated down-regulation of the major histocompatibility complex class I (MHC I) pathway-related genes occurred during the later stages of infection and appeared to be mediated by down-regulation of interferon-regulatory factor (IRF)-1, independent of interferon- $\alpha$ , interferon- $\gamma$  and IRF-2 expression. Within this micro-environment, suppression of the MHC I and possibly the MHC II pathways may inhibit the development of acquired immunity and could explain the unusually high susceptibility of Atlantic salmon to AGD.

## 6.2 Introduction

Amoebic gill disease (AGD) is an ectoparasitic condition of some marine fish (Dyková et al., 1995; Kent et al., 1988; Munday et al., 1990; Rodger and McArdle, 1996; Roubal et al., 1989). The only confirmed aetiological agent of AGD is the marine amoeba, *Neoparamoeba perurans* (Young et al., 2007) which is capable of infecting a number of fish species across a broad geographical range (Young et al., 2008c). Clinical signs of AGD include lethargy, respiratory distress and, if affected fish remain untreated, mortality (Kent et al., 1998; Munday et al., 1990). The characteristic gross sign of disease is restricted to the gills where multifocal lesions appear as pale gill tissue (Munday et al., 1990; Rodger and McArdle, 1996). Histological examination of these lesions reveals the hyperplasia of epithelial-like cells, resulting in extensive secondary lamellar fusion (Dyková et al., 1995; Kent et al., 1988; Munday et al., 1990; Rodger and McArdle, 1996; Roubal et al., 1989) and the progressive loss of normal gill structure (Adams and Nowak, 2003). Other than the focal proliferation of these epithelial-like cells, there is only a modest cellular response to *N. perurans* infection. A modest number of leucocytes migrate to the central venous sinus, adjacent to lesions and seldom are they seen within the lesions themselves (Adams and Nowak, 2001; Adams and Nowak, 2003).

Little is known about the host immune response to *N. perurans* infection, although some understanding has developed using a functional genomics (gene expression) approach. *N. perurans* induces interleukin-1 $\beta$  (IL-1 $\beta$ ) mRNA expression (Bridle et al., 2006a;

Bridle et al., 2006b) specifically in AGD-affected gill tissue (Morrison et al., 2007). However, modulation of IL-1 $\beta$  in AGD-affected tissue occurs without change in the expression of either tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) or inducible nitric oxide synthase (iNOS), genes typically activated in response to IL-1 $\beta$  (Laing et al., 2001). The apparent recalcitrance of these genes to IL-1 $\beta$ -induced modulation could be facilitated by the simultaneous down-regulation of the type I interleukin-1 receptor (Morrison et al., 2009 manuscript in preparation). On a more wide-spread scale, 190 unique transcripts were identified as being up or down-regulated in AGD-affected gill tissue and provided evidence that p53 potentially mediates the hyperproliferative response to *N. perurans* (Morrison et al., 2006a). Over progressive studies it has become apparent that the transcriptional response of individual genes is restricted to gill lesions, and the surrounding tissue remains unaffected (Bridle et al., 2006b; Morrison et al., 2006a; Morrison et al., 2009 manuscript in preparation; Morrison et al., 2007). Therefore, when examining AGD-affected gill tissue, a failure to specifically target AGD-affected tissue may have masked a specific transcriptional response (Morrison et al., 2006a).

To test this, we have utilised genomics resources developed by the Genomic Research on Atlantic salmon Project (GRASP) (Rise et al., 2004b) including a cDNA microarray (von Schalburg et al., 2005) to determine the biological mechanisms that mediate the susceptibility of Atlantic salmon to *N. perurans* induced AGD. In addition, we addressed the hypothesis that the transcriptional response may be restricted to the microenvironment within AGD lesions. Microarray and qRT-PCR analyses confirmed



that the majority of the transcriptional response to AGD was restricted to lesions. No evidence of a coordinated innate or adaptive immune response was observed, in fact AGD<sup>+</sup> lesions were characterised by a down-regulation in interferon- $\gamma$  (IFN- $\gamma$ ) and multiple IFN-inducible genes, particularly those involved in processing and presentation of antigens via the major histocompatibility complex class I (MHC I) and MHC class II (MHC II) pathways.

### **6.3 Materials and methods**

#### **6.3.1 *Neoparamoeba perurans* infection**

The harvesting of primary isolates of amoebae from the gills of AGD-affected Atlantic salmon and the experimental induction of AGD in Atlantic salmon were performed as described previously (Morrison et al., 2004). Amoebae were harvested from the gills of AGD-affected Atlantic salmon and assessed by light microscopy, PCR (Young et al., 2008b) and *in situ* hybridisation (ISH) using *N. perurans*, *Neoparamoeba pemaquidensis* and *Neoparamoeba branchiphila*-specific oligonucleotide probes (Young et al., 2007). Seawater-adapted Atlantic salmon (mean  $\pm$  standard error, 75.6  $\pm$  1.4 g, n = 20 fish/system) were placed into 6 $\times$  530 L autonomous recirculating tank systems. Three of the six recirculating systems were inoculated with amoebae at 500 cells/L and the systems were maintained at 15°C, 34 ppt salinity and pH 7.8 for 36 days. At 12, 25 and 36 days post-exposure (DPE) to *N. perurans*, all AGD-affected fish from one system and all AGD-naïve fish from one system were euthanised (5 mL/L Aqui-S NZ, Lower Hutt, New Zealand). The gills from ten fish per system were fixed in RNAlater RNA stabilisation reagent (Sigma–Aldrich, Castle Hill, New South Wales, Australia) at 4°C

overnight and stored at -20°C until required. The second, left anterior gill hemibranch and portions of head kidney, spleen, liver and intestine were removed from the remaining ten fish per system, fixed in Seawater Davidson's fixative for 24 h, processed, embedded in paraffin, sectioned (5 µm) and stained with haematoxylin and eosin following routine histological procedures. AGD gill lesions were identified by histological examination using the criteria described by Dyková and Novoa (2001). The severity of AGD was expressed as the proportion of gill filaments exhibiting AGD lesions within each section (Adams and Nowak, 2004b).

At day 36 DPE, gill hemibranchs were assessed using *in situ* hybridisation with *N. perurans*, *N. pemaquidensis* and *N. branchiphila*-specific probes as described above. In addition, primary isolates of amoebae were harvested from the gills of ten AGD-affected fish (Morrison et al., 2004) and the identity of gill-associated amoebae was verified by PCR using *N. perurans*-specific oligonucleotides as described above.

### **6.3.2 Amplification, labelling and hybridisation of RNA to microarray slides**

Experiments were designed to comply with the minimum information about a microarray experiment (MIAME) guidelines (Brazma et al., 2001). Amplified mRNA (aRNA) obtained from normal gill tissue from AGD-naïve fish (AGD<sup>-</sup> no lesion), gill lesions from AGD-affected fish (AGD<sup>+</sup> lesion) and “normal” gill tissue from AGD-affected fish (AGD<sup>+</sup> no lesion) was hybridised to a single print batch of the salmonid 16K Microarray Version 2.0 slides (von Schalburg et al., 2005). Paired samples were

labelled with different fluorophores before hybridising to the microarray slides and each biological replicate was reversed (dye flip) to compensate for cyanine fluorophore bias.

Gill tissue from fish sampled at 36 DPE to *N. perurans* was dissected under a stereomicroscope in order to isolate either normal tissue or AGD-affected tissue. AGD-affected tissue consisted of gill lesions from AGD-affected fish (AGD<sup>+</sup> lesion) while normal tissue from AGD-affected fish (AGD<sup>+</sup> no lesion) and AGD-naïve fish (AGD<sup>-</sup> no lesion) did not exhibit any evidence of hyperplastic change. Total RNA was purified from each sample using an RNeasy Mini Kit (Qiagen, Doncaster, Victoria, Australia) including an on-column DNase I (Qiagen) DNA digestion step according to the manufacturer's instructions. Total RNA concentrations were determined using a spectrophotometer and RNA integrity was verified by agarose gel electrophoresis.

To obtain adequate quantities of RNA for hybridisation experiments, 2 µg of total RNA from each fish was subjected to one round of amplification by using the MessageAmp aRNA Amplification Kit (Ambion, Scoresby, Victoria, Australia). The manufacturer's instructions were followed throughout except that dUTP was substituted with a mixture of amino allyl-dUTP (Sigma–Aldrich, Castle Hill, NSW, Australia) and dUTP at a ratio of 1:1 during RNA amplification. Dye coupling to aRNA was performed using either Cy3 or Cy5 according to the Amino Allyl MessageAMP Kit (Ambion) protocol with minor modifications. Briefly, each aRNA sample (3 µg) was dried to completion in a vacuum centrifuge, resuspended in coupling buffer (4.5 µL) and 5.5 µL of Cy3 or Cy5

(Amersham Biosciences, Castle Hill, NSW, Australia) was added for 30 min in darkness. To quench the reaction, 4 M hydroxylamine (2.25  $\mu$ L, Sigma-Aldrich) was added followed by the addition of nuclease-free water to a final volume of 30  $\mu$ L. Finally, dye-labelled aRNA was filter-purified according to the Amino Allyl MessageAMP Kit (Ambion) instructions.

All microarray slide pre-hybridisation procedures were as previously described (Morrison et al., 2006a). Labelled aRNA was hybridised to pre-warmed microarray slides in a formamide-based buffer (25% formamide, 4 $\times$  SSC, 0.5% SDS, 2 $\times$  Denhardt's solution and 4  $\mu$ L of Genisphere LNA dT blocker) for 16 h at 49°C. Post-hybridisation washes of the microarray slides were also as previously described (Morrison et al., 2006a). Images of the hybridised microarray slides were acquired immediately at 10  $\mu$ m resolution using a ScanArray Express (PerkinElmer, Fremont, California, USA) slide scanner. The Cy3 and Cy5 fluorophores were excited at 543 and 633 nm respectively and at the same laser power (90%). The photomultiplier tube settings were adjusted between slides to balance the Cy5 and Cy3 channels. Fluorescence intensity data were extracted from TIFF-formatted images using Imagen 5.6.2 software (BioDiscovery, El Segundo, California, USA).

Data analyses were performed in GeneSpring GX (Silicon Genetics, Redwood City, California, USA). For these analyses, background-corrected data were Lowess normalised (Yang et al., 2002) and only features with a signal intensity greater than or

equal to the average base/proportional value in the raw channel were retained. For the purposes of this study, features were incorporated into the data set if they were;

1. Significantly dysregulated in AGD-affected tissue as determined using a Student's *t*-test with Benjamini and Hochberg false discovery rate multiple test correction (MTCBH) (Benjamini and Hochberg, 1995) ( $p < 0.05$ ),
2.  $\geq 2$ -fold dysregulated, and
3. Satisfied criterion 1 and 2 in all biological replicates.

The raw data set has been deposited into the GEO (<http://www.ncbi.nlm.nih.gov/geo>, platform **GPL2716** and series **GS9595**).

The identification (ID) of features on the salmonid 16K Microarray Version 2.0 was initially assigned by the GRASP consortium (<http://web.uvic.ca/cbr/grasp>). The IDs of all features described in the current study were manually updated using the same process described by the GRASP consortium (von Schalburg et al., 2005). Briefly, Phrap-assembled contigs for each expressed sequence tag (EST) were updated with data from the ongoing GRASP consortium clustering project (Rise et al., 2004b). The ID of each contig was determined using BLASTX or BLASTN (Altschul et al., 1990) interrogation of non-redundant protein or nucleotide sequences in the GenBank database respectively (<http://www.ncbi.nlm.nih.gov/>). The IDs were filtered according to the criteria used by the GRASP consortium, so that the lowest-scoring significant hit ( $E < 10^{-15}$ ) in BLASTX was chosen to represent the EST; otherwise the lowest-scoring BLASTN hit was chosen to represent the EST.

### 6.3.3 RNA extraction and cDNA synthesis for quantitative real-time PCR (qRT-PCR)

For microarray data verification, 800 ng of AGD<sup>+</sup> lesion and AGD<sup>-</sup> no lesion aRNA from the microarray experiment was reverse transcribed using Superscript III reverse transcriptase and random hexamers (Invitrogen) according to the manufacturer's protocol. The aRNA cDNA template was diluted 80-fold to 1600 µL for further studies. Two undiluted samples of cDNA from AGD<sup>+</sup> lesion and AGD<sup>-</sup> no lesion aRNA were pooled in equal proportions and used as cDNA template to calculate the PCR amplification efficiency of each gene of interest.

Total RNA was isolated from AGD<sup>+</sup> lesion, AGD<sup>+</sup> no lesion and AGD<sup>-</sup> no lesion RNAlater-stabilised gill tissues sampled at 12, 25 and 36 DPE to *N. perurans*. Total RNA was purified using an RNeasy Mini Kit (Qiagen), a Dounce homogeniser (Wheaton Scientific, Millville, NJ) and QIAshredders (Qiagen). All RNA was DNase I-treated using Turbo DNA-free (Ambion). RNA extraction and DNase I treatment were performed according to the manufacturer's instructions. The quality and quantity of total RNA was measured as described above. To determine the relative expression of genes at various times post-exposure to *N. perurans*, total RNA was reversed transcribed from samples obtained at 12 (120 ng), 25 (800 ng) and 36 (800 ng) DPE as described above except random hexamers were replaced by a mix of ten parts random hexamers (Invitrogen) (50 ng) and one part oligo(dT)<sub>20</sub> (Invitrogen) (1 µL of 50 µM stock) as

advised by Quantace (Neutral Bay, New South Wales, Australia). cDNAs were diluted 3-fold (12 DPE) or 8-fold (25 and 36 DPE) for further studies. Three undiluted samples of cDNA from AGD<sup>+</sup> lesion and AGD<sup>-</sup> no lesion 36 DPE to *N. perurans* were pooled in equal proportions and used as cDNA template to calculate the amplification efficiency of each gene of interest.

#### **6.3.4 qRT-PCR assays**

A select number of differentially expressed genes identified during microarray analyses were verified by qRT-PCR (Table 6.1). In addition, the relative expression of mRNA from genes involved in the MHC class I antigen presentation pathway at 12, 25 and 36 DPE to *N. perurans* was confirmed by qRT-PCR. PCR oligonucleotides were designed against Atlantic salmon transcripts of interest and to anneal at 55°C (Table 6.1). When more than one transcribed locus existed for genes that played a similar functional role, oligonucleotides were designed to be pan-specific for transcripts encoded by those genes. In some cases, oligonucleotide sequences were available for genes including  $\beta$ -actin (Morrison et al., 2006a), RNA polymerase II (RPL2) (Jørgensen et al., 2006a) and MHC class I (Jørgensen et al., 2006a). In the case of the MHC class I gene, the oligonucleotides amplified a portion of the conserved  $\alpha 3$ -region of the *Sasa*-UBA locus.

Table 6.1. Sequences of oligonucleotides used to determine relative expression of genes by quantitative real-time PCR

Reference/ Accession Number <sup>a</sup>	Gene identification of top BLAST hit <sup>b</sup>	qRT-PCR oligonucleotide sequences (5' - 3') F- forward, R- reverse oligonucleotide	Amplicon (bp)
<b><u>S77332</u></b>	Ferritin heavy subunit <sup>c,d,e</sup>	F- CAGAGGGGAGGGAGAATC R- AGGGACTGGTTCACACTC	119
Morrison et al. 2006a	$\beta$ -actin <sup>e</sup>	F- TCTCTGGAGAAGAGCTAC R- CAAGACTCCATACCGAGGAA	158
<b><u>AF321836</u></b>	Elongation factor 1 $\alpha$ (EF1 $\alpha$ ) <sup>c,d,e</sup>	F- TGATTGTGCTGTGCTTATCG R- AACGCTTCTGGCTGTAGG	173
Jorgensen et al. 2006a	RNA polymerase II (RPL2) <sup>c,d,e</sup>	F- TAACGCCTGCCTCTTCACGTTGA R- ATGAGGGACCTTGTAGCCAGCAA	112
Jorgensen et al. 2006a	MHC I $\alpha$ 3 <sup>c,d</sup>	F- CTGCATTGAGTGGCTGAAGA R- GGTGATCTGTCCGTCTTTC	176
<b><u>AF180487</u></b>	$\beta$ 2-microglobulin ( $\beta$ 2m) <sup>c,d</sup>	F- TCCCAGACGCCAAGCAG R- TGTAGGTCTTCAGATTCTTCAGG	138
<b><u>DQ451008</u></b>	TAP binding protein (TAPBP) <sup>c,d</sup>	F- GTCCCTCTCCATCTTCCC R- CCTGACGCCTCGCATTG	78
<b><u>CB501462</u></b>	Proteasome activator subunit 2 $\beta$ (PA28 $\beta$ ) <sup>c</sup>	F- GCAGTGTCTAAAGCCTCCAAG R- CATAAGTTCGCATAGAAGCC	133
<b><u>CA063863</u></b>	Interferon regulatory factor 1 (IRF-1) <sup>d</sup>	F- ACAGTCAAGAGAGCCAATG R- CCAGTAGTCGGTGTAAGAG	119
<b><u>AJ841811</u></b>	Interferon- $\gamma$ (IFN- $\gamma$ ) <sup>d</sup>	F- GGCTCTGTCCGAGTTCATTACC R- GGGCTTGCCGTCTCTTCC	98
<b><u>X70165</u></b>	MHC II $\beta$ <sup>c</sup>	F- ACCCGTCCCTGCCTGAG R- TGAGTAGATGAGTCCTGCCAAG	99
<b><u>CK990275</u></b>	Invariant chain (Iclp) <sup>c</sup>	F- GCCACAGTTCAACGAGAC R- CTCAAAGCCCTCCCACTC	82
<b><u>CK990940</u></b>	C1q-like adipose specific protein (C1q-like) <sup>c</sup>	F- AGGACCATACAATACTGACATCACC R- CCGAAGTAGTAGATTCCACTCACC	120
<b><u>CB511650</u></b>	Keratin type I S8 (Keratin I S8) <sup>c</sup>	F- CTCGGGCTACCAGAACCAG R- TCCAGCAGCATCTTGTAAATCG	99
<b><u>AY572832</u></b>	C-type lectin receptor A (CLRA) <sup>c</sup>	F- GCCGTTACCTAGTGTCCC R- CACTTCCTTACTGTCTTTGAGC	132

<sup>a</sup> GenBank (<http://www.ncbi.nlm.nih.gov/>) nucleotide accession number used for oligonucleotide design.

<sup>b</sup> Basic local alignment search tool (BLAST).

<sup>c</sup> Genes used to verify aRNA microarray relative expression.

<sup>d</sup> Genes investigated for change in relative expression of mRNA sampled at 12, 25 and 36 days post-exposure to *Neoparamoeba perurans*.

<sup>e</sup> Reference genes used to normalise relative expression values.



PCR amplification was performed using a real-time PCR detection system (IQ5, Bio-Rad Laboratories, Gladesville, New South Wales, Australia). Each reaction consisted of template cDNA (2  $\mu$ L), 2 $\times$  SensiMixPlus SYBR & Fluorescein (11  $\mu$ L, Quantace), gene-specific oligonucleotides (0.2  $\mu$ M each) and nuclease-free water to a final volume of 22  $\mu$ L. All cDNAs were amplified in duplicate using the following thermal cycling parameters: 1 cycle of 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 55°C for 30 sec and 72°C for 25 sec. In addition, duplicate reverse transcriptase-free cDNA synthesis reaction and no template controls were performed for each oligonucleotide pair. qRT-PCR amplification efficiencies were calculated using five 10-fold (aRNA qRT-PCR) or 5-fold (mRNA qRT-PCR) cDNA template dilutions.

All qRT-PCR reactions were subjected to post-amplification melt-curve analysis and a PCR amplicon from each gene examined was analysed by gel electrophoresis and nucleotide sequencing. The methods used to ligate PCR products into a plasmid vector, transformation of *E. coli*, colony selection and plasmid purification were as previously described (Young et al., 2007). Sequencing reactions were initiated using an M13 reverse oligonucleotide (22-mer, Promega, Annandale, NSW, Australia) and analysed on an ABI 3730xl DNA analyser (Applied Biosystems (ABI), Scoresby, Victoria, Australia).

### 6.3.5 qRT-PCR data analysis

Baseline level and cycle threshold ( $C_T$ ) were set automatically on the real-time PCR detection system (IQ5, Bio-Rad). The mean  $C_T$  deviation between the treatment and control groups was determined and normalised against multiple reference genes (Table 6.1). Reference genes were either previously validated for real-time PCR in Atlantic salmon (Jørgensen et al., 2006a; Morrison et al., 2006a) or found to be stable across samples analysed during the microarray experiment. A total of four reference genes were assessed by qRT-PCR and their expression was not significantly affected by AGD (data not shown). The relative stability of reference genes was calculated as a gene expression stability measure ( $M$ ) using GeNorm software (Vandesompele et al., 2002). Reference genes with the three lowest  $M$  values [ferritin heavy subunit, elongation factor 1 $\alpha$  (EF1 $\alpha$ ) and RPL2] were used to normalise the relative expression values during qRT-PCR analyses (Table 6.1). The relative expression (fold change) of each gene of interest was determined after empirically-derived PCR efficiencies were used to correct the data using Relative Expression Software Tool software (REST-384 version 2) (Pfaffl et al., 2002). The relative expression of the genes of interest was subsequently tested for significance ( $p = 0.05$ ) by a pair-wise fixed reallocation randomisation test using 2000 randomisations (Pfaffl et al., 2002).

## 6.4 Results

### 6.4.1 Induction of AGD in Atlantic salmon and taxonomic identification of the aetiological agent

*N. perurans* was the only detectable amoeba in the inoculum used to initiate AGD and on the gills of AGD-affected Atlantic salmon. These are important data since there is no pathogenic clonally-cultured *Neoparamoeba* sp. currently available and the only source of virulent cells is fish infected by cohabitation. Moreover, while it has recently been shown that *N. perurans* is an agent of AGD (Young et al., 2007) the role of *N. pemaquidensis* (Kent et al., 1988) and *N. branchiphila* (Dyková et al., 2005b) in AGD is yet to be resolved. Initially, amoebae used to inoculate tanks housing Atlantic salmon was screened for *N. perurans* by PCR (Fig. 6.1A) followed by *in situ* hybridisation (ISH, Fig. 6.1B). *N. perurans* was the only detectable *Neoparamoeba* species and no evidence of either *N. pemaquidensis* or *N. branchiphila* was found. At all sampling points, gross examination of the gills from AGD-naïve fish revealed a normal structure with no visible hyperplastic tissue (Fig. 6.1C). At 12 DPE to *N. perurans*, fish displayed only a few focal, raised patches of gill tissue, typical of AGD-like lesions (data not shown). There was a clear temporal change in the severity of AGD, with numerous AGD-like lesions distinguishable across the gills of fish at 25 and 36 DPE (Fig. 6.1D) to *N. perurans*.

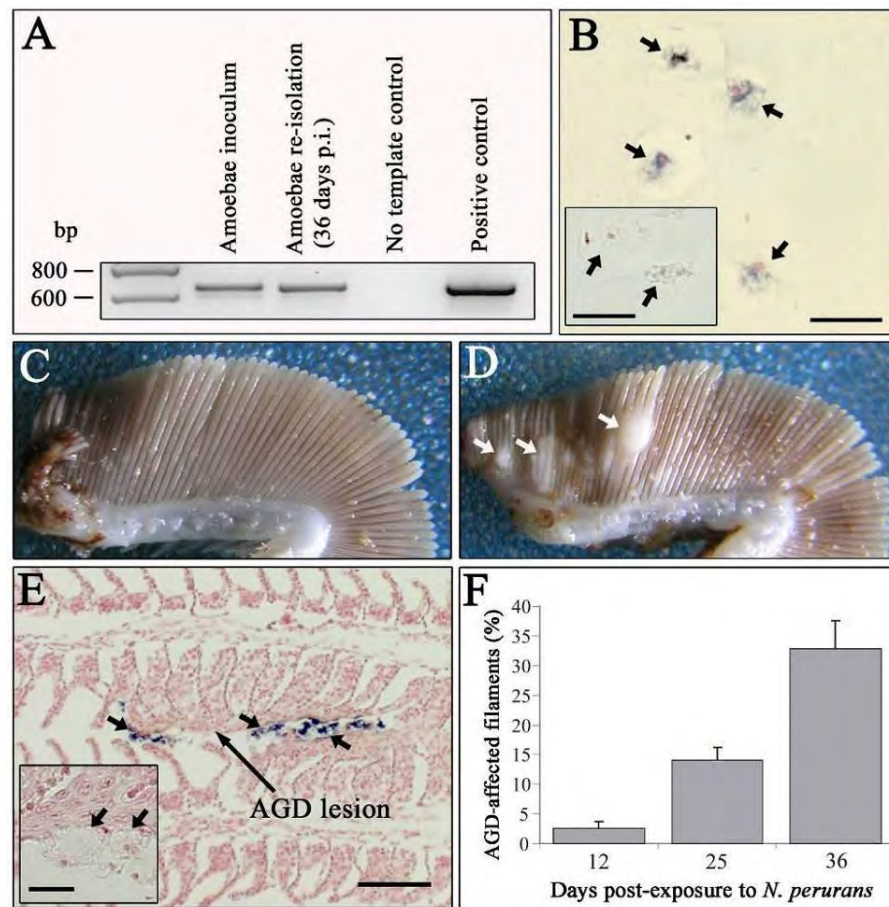


Figure 6.1. Confirmation that Atlantic salmon were affected by *Neoparamoeba perurans*-induced amoebic gill disease (AGD). (A) Detection of *N. perurans* rRNA gene by PCR in both the amoebae inoculum used to induce AGD and in primary isolates of amoebae obtained from AGD-affected fish at the conclusion of the experiment (36 days post-inoculation). (B) Confirmation that all amoebae (arrows) in the inoculum used to induce AGD were *N. perurans*. Amoeba (arrows) identity was assessed by *in situ* hybridisation using an *N. perurans*-specific oligonucleotide probe. Bar = 25  $\mu$ m. Insert shows amoebae assessed using a *Neoparamoeba pemaquidensis*-specific oligonucleotide probe as a negative control. Bar = 25  $\mu$ m. (C) Seawater Davidson's-fixed gill arch from an AGD-naïve fish. (D) Seawater Davidson's-fixed gill arch from a fish at 36 days post-exposure (DPE) to *N. perurans* showing prominent, focal AGD-like lesions (arrows). (E) Confirmation that AGD was induced in fish at 36 DPE to *N. perurans*. Amoebae (arrows) identity was assessed by *in situ* hybridisation using an *N. perurans*-specific oligonucleotide probe. Bar = 100  $\mu$ m. Insert shows amoebae assessed using a *N. pemaquidensis*-specific oligonucleotide probe as a negative control. Bar = 20  $\mu$ m. (F) Temporal change in the proportion of AGD-affected gill filaments (mean + standard error, n = 10 fish).

Structural changes in gill tissue were examined histologically and were consistent with the pathology described in other cases of AGD (Dyková et al., 1995; Kent et al., 1988; Munday et al., 2001; Rodger and McArdle, 1996; Roubal et al., 1989). The most prominent feature was the extensive hyperplasia of cells, resulting in fusion of the secondary lamellae (Fig. 6.1E). At the conclusion of the experiment (36 DPE to *N. perurans*), amoebae harvested from the gills of AGD-affected fish were screened by PCR and the presence of *N. perurans* was verified (Fig 6.1A). In gill tissues examined from three fish at 36 DPE, only the *N. perurans*-specific ISH probe hybridised with the amoebae associated with AGD-lesions (Fig. 6.1E). On serially-sectioned gill tissue from the same fish, neither the *N. pemaquidensis*-specific probe (Fig. 6.1E inset) nor the *N. branchiphila*-specific probe (data not shown) hybridised with amoebae present in gill tissue.

Histological evidence of AGD in gill tissues of fish exposed to *N. perurans* was apparent at each sampling point over the time-course study. A step-wise increase in the proportion of AGD-affected gill filaments was evident, resulting in  $32.9 \pm 4.7\%$  (mean  $\pm$  SEM) of gill filaments affected by AGD at 36 DPE to *N. perurans* (Fig. 6.1F). It is noteworthy that this proportion of AGD-affected filaments is consistent with that observed in cases of AGD in sea-cage cultured Atlantic salmon in Tasmania, Australia (Adams and Nowak, 2003) indicating that data presented here are of relevance to both tank and field-based *N. perurans* infections.

## **6.4.2 Profile of the effects of AGD on lesion and non-lesion gill tissue gene expression**

### **6.4.2.1 Overall transcriptional response by gene category**

Two microarray studies were conducted to identify transcripts that were differentially-regulated in AGD-affected gill tissue and secondarily, to determine whether gene dysregulation was restricted to AGD lesions (Fig. 6.2). When gene expression in AGD<sup>+</sup> lesion tissue was compared to that in AGD<sup>-</sup> no lesion tissue (n = 6 biological replicates), 340 features were significantly and reproducibly dysregulated (Student's *t*-test  $p < 0.05$ , dysregulated by  $\geq 2$ -fold and MTCBH corrected) in all biological and technical replicate hybridisations (Appendix 1: Supplementary Table 1). Similarly, when gene expression in AGD<sup>+</sup> lesion tissue was compared to that in AGD<sup>+</sup> no lesion tissue (n = 4 biological replicates), 333 features were significantly and reproducibly dysregulated. Of these 333 features, a total of 233 features were shared across both microarray studies (Appendix 1: Supplementary Table 1). Often, multiple features represented the same transcript and in these instances, a representative feature is provided (Table 6.2). Features with no significant BLAST hit ( $E > 1e^{-15}$ ) were designated unknown (74 features, 29.6% of total features, Fig. 6.3) and removed from the summarised gene lists (Table 6.2). For a full list of differentially regulated genes see Supplementary Tables 1 and 2 (Appendix 1).

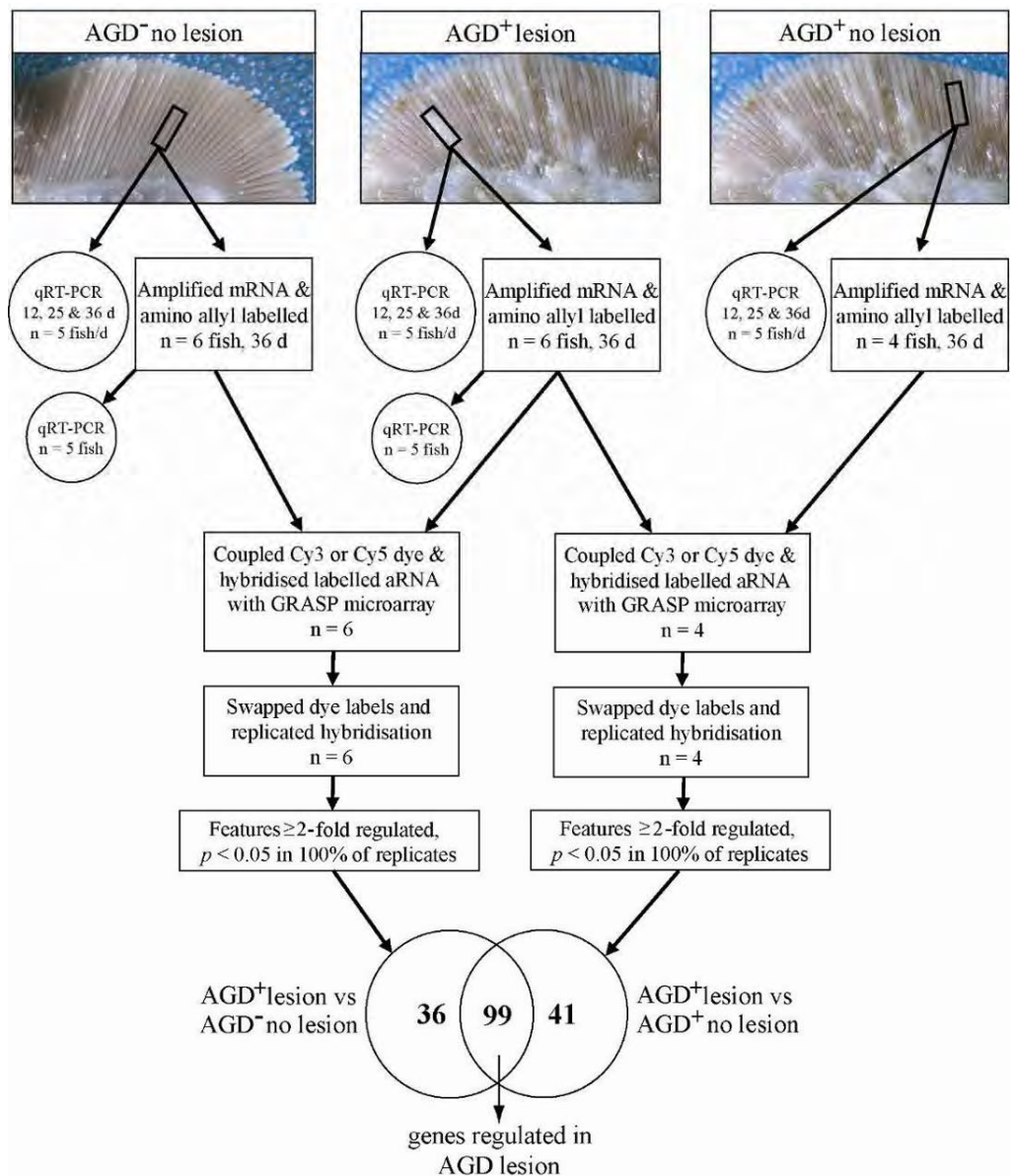


Figure 6.2. Overview of the microarray and quantitative real-time PCR (qRT-PCR) studies. Total RNA was extracted from healthy, amoebic gill disease (AGD)-naïve Atlantic salmon (AGD<sup>-</sup> no lesion), AGD-affected gill lesion tissue (AGD<sup>+</sup> lesion) and “normal” gill tissue from the same gill arch as the AGD-affected tissue (AGD<sup>+</sup> no lesion). A total of 20 hybridisations were performed, which included the technical replication of each pair of biological replicates (Cy3-Cy5 dye-swap). Venn diagram shows overlap between the AGD<sup>+</sup> lesion vs. AGD<sup>-</sup> no lesion and AGD<sup>+</sup> lesion vs. AGD<sup>+</sup> no lesion tissue comparisons with the dysregulation of 99 non-redundant, annotated transcripts shared across microarray studies.

After the removal of redundant and unknown transcripts, a total of 135 annotated transcripts were differentially-regulated in AGD<sup>+</sup> lesion compared to AGD<sup>-</sup> no lesion tissue and 140 differentially-regulated in AGD<sup>+</sup> lesion compared to AGD<sup>+</sup> no lesion tissue. Of these genes, 99 annotated transcripts were common to both microarray studies and thus represented AGD<sup>+</sup> lesion-restricted dysregulated genes (Fig. 6.2).

The complete sets of annotated, non-redundant genes differentially-regulated across both microarray studies are provided in the supplementary material (Supplementary Table 2). Annotated transcripts not common to both microarray studies predominantly belonged to two groups: (1) Transcripts that did not strictly conform to the criteria used to identify features such that they were not significantly dysregulated in all biological and technical replicates in both microarray studies or (2) Genes dysregulated across the entire gill arch. The differentially-regulated genes were categorised according to their function using Gene Ontology (GO, <http://www.geneontology.org/>) annotations within the Uniprot (<http://beta.uniprot.org/>), ExPASy (<http://au.expasy.org/>) and NCBI (<http://www.ncbi.nlm.nih.gov/>) databases. Genes were then grouped into higher-order functional categories within their respective parental GO terms (Fig. 6.3). The most populated functional categories that were differentially-regulated in response to AGD were metabolic processes (13.2% of total genes) and immune response (9.6% of total genes) (Fig. 6.3). In addition, a significant proportion of genes (13.6% of total genes) had no GO annotation. To illustrate trends in gene dysregulation, the induction/suppression of genes within each functional category was displayed on a



colour-coded gene regulation scale (Fig. 6.3). In general, when genes were dysregulated in both microarray studies, the patterns of expression were consistent. A strong overall pattern of gene suppression was observed in the immune response, transport, translation and catalytic activity categories while other categories were populated by an approximately equal abundance of up and down-regulated genes (Fig. 6.3).

#### **6.4.2.2 Dysregulation of the immune response in AGD lesions from affected**

##### **Atlantic salmon**

Within the immune response category described above, the majority of genes were down-regulated, indicating that *N. perurans* succeed in limiting the host immune response in AGD<sup>+</sup> lesions (Table 6.2, bold text). The down-regulated transcripts were predominantly associated with antigen processing and presentation through the MHC I or MHC II pathways. Atlantic salmon transcripts similar to mammalian genes that play a role in processing and presenting cytosolic antigens via the MHC I pathway included the proteasome activator subunit 2- $\beta$  (PA28 $\beta$ ), a subunit of the membrane bound transport-associated with antigen processing protein (TAP2B), the MHC I and  $\beta$ -2-microglobulin ( $\beta$ 2m) molecules that form the MHC I complex and TAP binding protein (TAPBP).

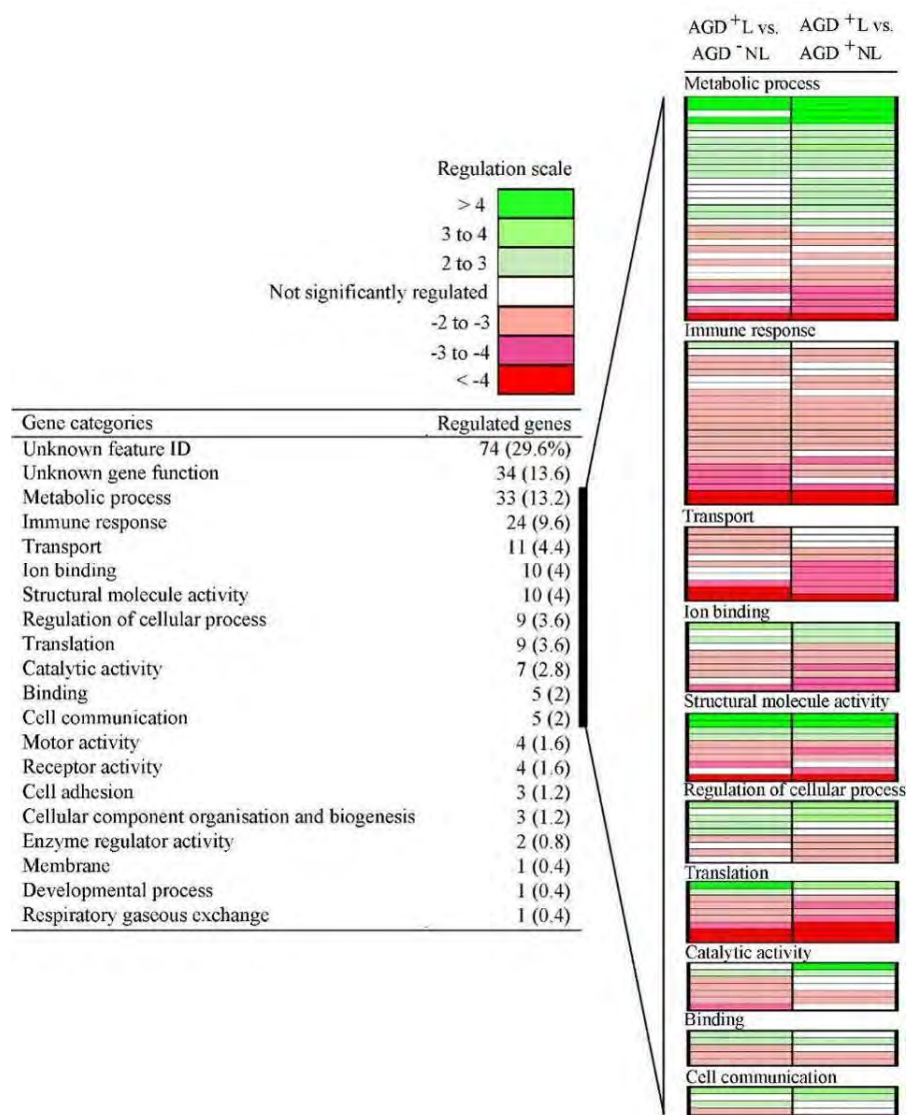


Figure 6.3. Gene categories containing representatives that were differentially-regulated in the microarray studies. The number of representatives and the percentage of the total non-redundant genes are shown. Values in parentheses are the percentage of genes within each category of the total non-redundant genes that were differentially-regulated. The expression profile of each category with five or more representatives is shown. A gene regulated in the AGD<sup>+</sup> lesion relative to AGD<sup>-</sup> no lesion microarray study is shown next to the same gene in the AGD<sup>+</sup> lesion relative to AGD<sup>+</sup> no lesion study. Changes in the level of gene expression were analysed by Student's *t*-test ( $p < 0.05$ ) and the expression ratio is coded with a colour scale. Within each category, the genes were ranked from most up-, to most down-regulated. For the list of all genes and individual gene regulation values see Supplementary Tables 1 and 2.

Atlantic salmon transcripts corresponding to the type I and type II  $\beta 2m$  molecules of rainbow trout (*Oncorhynchus mykiss*) (Magor et al., 2004; Shum et al., 1996) were down-regulated in AGD<sup>+</sup> lesions. In addition, a single down-regulated feature with a BLASTX hit to  $\beta 2m$  of the channel catfish (*Ictalurus punctatus*, 98.7% amino acid identity) also shared 98.7% deduced amino acid sequence identity with the putative type I  $\beta 2m$  of Atlantic salmon (data not shown) and most likely represents the latter. Atlantic salmon possess multiple MHC I loci (Lukacs et al., 2007; Miller et al., 2006). Only the *Sasa*-UBA locus, found within the MHC I A gene region (MHC IA), retains characteristics of functional MHC I molecules (Grimholt et al., 2002; Lukacs et al., 2007) and was also down-regulated in AGD<sup>+</sup> lesions (18 significantly dysregulated features). Another MHC I molecule down-regulated in AGD<sup>+</sup> lesions was the transcript encoded by the MHC IA/IB-independent *Sasa*-ZE locus (Lukacs et al., 2007).

Table 6.2. Subset of non-redundant, annotated features differentially-regulated in the gills of AGD-affected Atlantic salmon.

Assembled contig top BLAST hit <sup>a</sup>	E value <sup>†</sup>	Specific gene ontology terms <sup>b</sup>	EST accession number <sup>c</sup>	AGD <sup>+</sup> L vs. AGD <sup>-</sup> NL fold change <sup>d</sup>	AGD <sup>+</sup> L vs. AGD <sup>+</sup> NL fold change
<i>Immune response<sup>e</sup></i>					
Cell surface glycoprotein CD53 (NP_000551; <i>Homo sapiens</i> )	3e-40	signal transduction GO:0007165	CA051581		-2.00 ± 0.22
Chemokine CC-like protein mRNA (DQ339045; <i>Scophthalmus maximus</i> )*	4e-24	chemokine activity GO:0008009	CB509719	2.42 ± 0.17	
Complement component 1, q subcomponent, $\gamma$ polypeptide (EDL80826; <i>Rattus norvegicus</i> )	3e-16	phosphate transport GO:0006817, tumour necrosis factor receptor binding GO:0005164	CB501016	-2.21 ± 0.17	
Guanylate-binding protein 1 (AAM44075; <i>Oncorhynchus mykiss</i> )	5e-26	GTP binding GO:0005525	CB500029		-2.26 ± 0.25
IgM heavy chain C region (A46533; <i>Salmo salar</i> )	0	immune response GO:0006955	CA054828	-2.82 ± 0.44	-2.49 ± 0.17
IgT heavy chain A locus (AY872256; <i>O. mykiss</i> )*	4e-18		CA050554	-3.03 ± 0.33	-2.72 ± 0.41
Interferon regulatory factor 1 (AAM77843; <i>O. mykiss</i> )	1e-124	regulation of transcription GO:0045449, transcription factor activity GO:0003700	CA063863 (4)	-2.86 ± 0.66	
Invariant chain-like protein 1, INVX (AAL58577; <i>O. mykiss</i> )	6e-101	antigen processing and presentation GO:0019882, MHC class II protein binding GO:0042289	CK990275 (3)	-2.50 ± 0.47	-2.19 ± 0.28
Invariant chain-like protein 1, S25-7 (AAL91668; <i>O. mykiss</i> )	1e-144	antigen processing and presentation GO:0019882, MHC class II protein binding GO:0042289	CA769983 (12)	-3.08 ± 0.57	-2.24 ± 0.27
Invariant chain-like protein 2, 14-1 (AAL58575; <i>O. mykiss</i> )	4e-97	antigen processing and presentation GO:0019882, MHC class II protein binding GO:0042289	CB511842 (5)	-2.83 ± 0.41	-2.40 ± 0.14
MHC class I Onmy-UGA (AAP04358; <i>O. mykiss</i> )	3e-115	antigen processing and presentation GO:0019882	CK990213 (5)	-2.35 ± 0.35	
MHC class I Sasa-UBA (AAN75107; <i>S. salar</i> )	0	antigen processing and presentation GO:0019882	CA057048 (18)	-3.41 ± 0.7	-3.00 ± 0.50
MHC class I Sasa-ZE (AAZ76730; <i>S. salar</i> )	2e-172	antigen processing and presentation GO:0019882	CA040508 (2)	-2.62 ± 0.41	-2.03 ± 0.27
MHC class II Sasa-DAA (CAD27723; <i>S. salar</i> )	4e-124	antigen processing and presentation GO:0019882	CN442518 (4)	-2.36 ± 0.37	-2.44 ± 0.20
MHC class II Sasa-DAB (CAA49726; <i>S. salar</i> )	8e-128	antigen processing and presentation GO:0019882	CN442529 (9)	-2.58 ± 0.55	-2.72 ± 0.43
Proteasome activator subunit 2 $\beta$ (PA28) (NP_571449; <i>Danio rerio</i> )	2e-88	proteasome activator activity GO:0008538	CB501462	-5.06 ± 1.52	-8.10 ± 2.50
T cell receptor $\alpha$ chain (AAS79491; <i>S. salar</i> )	1e-73	receptor activity GO:0004872	CB516976	-2.22 ± 0.42	
Tapasin b (TAPBPb) pseudogene OSU-142 (DQ092330; <i>O. mykiss</i> )*	0	antigen processing and presentation of endogenous peptide antigen via MHC class I GO:0019885	CA064342		-2.26 ± 0.42
Transport associated protein TAP2B mRNA (Z83329; <i>S. salar</i> )*	1e-67	ATPase activity, coupled to transmembrane movement of substances GO:0042626	CK990475	-2.96 ± 0.47	-3.91 ± 0.85
$\beta$ 2-microglobulin putative type I (AAG17525; <i>S. salar</i> )	2e-60	antigen processing and presentation of peptide antigen via MHC class I GO:0002474	CB500803 (14)	-2.57 ± 0.44	
$\beta$ 2-microglobulin putative type II (AAG17531; <i>S. salar</i> )	1e-63	antigen processing and presentation of peptide antigen via MHC class I GO:0002474	CB505897 (6)	-2.07 ± 0.29	-2.09 ± 0.33
$\beta$ 2-microglobulin precursor (O42197; <i>Ictalurus punctatus</i> )	2e-42	antigen processing and presentation of peptide antigen via MHC class I GO:0002474	CK990626	-2.45 ± 0.40	-2.42 ± 0.41
<i>Metabolic process</i>					
Arachidonate lipoxygenase 3 (AAI16260; <i>Mus musculus</i> )	1e-86	leukotriene metabolic process GO:0006691, lipoxygenase activity GO:0016165	CA058392	8.88 ± 1.58	5.68 ± 1.64
Matrix metalloproteinase 13 (BAB19131; <i>O. mykiss</i> )	2e-41	proteolysis GO:0006508, metalloproteinase activity GO:0008237	CK990871 (3)	2.14 ± 0.40	2.63 ± 0.40
Nephrosin (BAE97360; <i>Plecoglossus altivelis altivelis</i> )	8e-110	proteolysis GO:0006508, metalloproteinase activity GO:0008237	CB510964 (3)		-2.24 ± 0.26
Tyrosine-protein kinase SRK2 (P42688; <i>Spongilla lacustris</i> )	1e-47	protein amino acid phosphorylation GO:0006468, protein-tyrosine kinase activity GO:0004713	CB514625	-3.83 ± 0.50	-3.23 ± 0.64
Vacuolar ATP synthase subunit S1 precursor (P40682; <i>Bos taurus</i> )	2e-19	ATP synthesis coupled proton transport GO:0015986, hydrogen ion transporting ATPase activity, rotational mechanism GO:0046961	CA056920	2.90 ± 0.26	2.29 ± 0.31

Assembled contig top BLAST hit <sup>a</sup>	E value <sup>†</sup>	Specific gene ontology terms <sup>b</sup>	EST accession number <sup>c</sup>	AGD <sup>+</sup> L vs AGD <sup>-</sup> NL fold change <sup>d</sup>	AGD <sup>+</sup> L vs AGD <sup>+</sup> NL fold change
<i>Structural molecule activity</i>					
Claudin 23a (AAT64074; <i>Takifugu rubripes</i> )	4e-26	structural molecule activity GO:0005198	CB510873	-2.39 ± 0.29	-2.88 ± 0.43
Collagen type I α 1 (BAB55661; <i>O. mykiss</i> )	4e-66	phosphate transport GO:0006817, extracellular matrix structural constituent GO:0005201	CB494364 (6)	-2.21 ± 0.18	-2.49 ± 0.42
Collagen type I α 2 precursor (O93484; <i>O. mykiss</i> )	0	phosphate transport GO:0006817, extracellular matrix structural constituent GO:0005201	CB486593 (3)	-2.32 ± 0.41	-3.79 ± 0.38
Collagen type I α 3 (BAB55662; <i>O. mykiss</i> )	1e-161	phosphate transport GO:0006817, extracellular matrix structural constituent GO:0005201	CA061635 (3)	-3.17 ± 0.57	
Keratin type I <sub>S</sub> S8, <i>Onmy</i> -K10 (CAC45059; <i>O. mykiss</i> )	1e-176	structural molecule activity GO:0005198	CB511650 (4)	6.88 ± 0.81	5.23 ± 0.72
Keratin type I <sub>S</sub> , <i>Onmy</i> -K18 (CAA74664; <i>O. mykiss</i> )	4e-141	structural molecule activity GO:0005198	CA042066 (6)	6.19 ± 0.55	4.34 ± 0.23
Keratin type II <sub>E</sub> E1, <i>Onmy</i> -K1 (AJ272369; <i>O. mykiss</i> )*	1e-129	structural molecule activity GO:0005198	CB510503	2.33 ± 0.19	2.20 ± 0.30
Keratin type II <sub>E</sub> E3, <i>Onmy</i> -K3 (CAC87008; <i>O. mykiss</i> )	0	structural molecule activity GO:0005198	CB492926 (6)	2.87 ± 0.24	2.45 ± 0.28
<i>Regulation of cellular process</i>					
CCAAT/enhancer-binding protein β (AAN41660; <i>O. mykiss</i> )	2e-140	regulation of transcription, DNA-dependent GO:0006355	CA055521	3.02 ± 0.53	3.30 ± 0.44
CCAAT/enhancer-binding protein β2 (ABD84407; <i>O. mykiss</i> )	4e-141	regulation of transcription, DNA-dependent GO:0006355	CA055219	2.36 ± 0.62	3.03 ± 0.22
Inhibitor of differentiation 1C; ID1C (AAQ55174; <i>O. mykiss</i> )	4e-65	regulation of transcription GO:0045449, transcription regulator activity GO:0030528	CA052765	2.13 ± 0.19	
Inhibitor of differentiation 2A ID2A (CAA69657; <i>O. mykiss</i> )	4e-69	regulation of transcription GO:0045449, transcription regulator activity GO:0030528	CB510736		-2.24 ± 0.14
JunB protein (AAU81662; <i>O. mykiss</i> )	4e-160	regulation of transcription GO:0045449, transcription factor activity GO:0003700	CA054491 (3)		2.88 ± 0.29
<i>Transport</i>					
Hemoglobin subunit α (P11251; <i>S. salar</i> )	4e-75	oxygen transport GO:0015671, oxygen transporter activity GO:0005344	CA061084 (9)	-2.79 ± 1.13	-3.22 ± 0.19
Hemoglobin, subunit β (CAA65950; <i>S. salar</i> )	4e-79	oxygen transport GO:0015671, oxygen transporter activity GO:0005344	CK990870 (17)	-3.21 ± 1.66	-3.69 ± 0.31
Na <sup>+</sup> /K <sup>+</sup> ATPase α subunit isoform 1b (AAQ82789; <i>O. mykiss</i> )	3e-74	monovalent inorganic cation transport GO:0015672, ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism GO:0015662	CK990492 (2)	-4.84 ± 0.42	-3.57 ± 0.60
Na <sup>+</sup> /K <sup>+</sup> ATPase α subunit isoform 1c (AAQ82788; <i>O. mykiss</i> )	0	monovalent inorganic cation transport GO:0015672, ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism GO:0015662	CA054630	-2.28 ± 0.15	
Na <sup>+</sup> /K <sup>+</sup> ATPase β subunit (AAT48994; <i>Rhabdosargus sarba</i> )	5e-125	monovalent inorganic cation transport GO:0015672, sodium:potassium-exchanging ATPase activity GO:0005391	CA062436 (2)	-6.17 ± 0.52	-4.2 ± 0.47
<i>Ion binding<sup>e</sup></i>					
Allograft inflammatory factor 1 (CAD45006; <i>T. rubripes</i> )	4e-62	calcium ion binding GO:0005509	CB489425		-2.10 ± 0.28
Annexin A1a (AAU81665; <i>O. mykiss</i> )	0	calcium-dependent phospholipid binding GO:0005544	CA054909	2.07 ± 0.25	2.15 ± 0.18
Kruppel-like factor 11 (NP_001006417; <i>Gallus gallus</i> )	4e-73	Zinc ion binding GO:0008270	CB516494		-3.05 ± 0.32
Osteonectin, Secreted protein, acidic, rich in cysteine SPARC (AAP04488; <i>Sparus aurata</i> )	3e-135	calcium ion binding GO:0005509	CB492428 (7)	-2.54 ± 0.25	-3.71 ± 0.67
S100 calcium binding protein (CAJ90906; <i>S. salar</i> )	7e-33	calcium ion binding GO:0005509	CB503763 (8)	3.48 ± 0.34	2.72 ± 0.46
<i>Receptor activity</i>					
C type lectin receptor A (AAT77220; <i>S. salar</i> )	8e-63	sugar binding GO:0005529	CB516930		3.53 ± 0.82
C type lectin receptor B (AAT77221; <i>S. salar</i> )	3e-51	sugar binding GO:0005529	CB500040	-2.09 ± 0.20	-2.13 ± 0.25

Assembled contig top BLAST hit <sup>a</sup>	E value <sup>†</sup>	Specific gene ontology terms <sup>b</sup>	EST accession number <sup>c</sup>	AGD <sup>+</sup> L vs AGD <sup>-</sup> NL fold change <sup>d</sup>	AGD <sup>+</sup> L vs AGD <sup>+</sup> NL fold change
<i>Motor activity</i>					
Myosin heavy chain (BAC00871; <i>Oncorhynchus keta</i> )	2e-94	ATP binding GO:0005524	CB497013	3.02 ± 0.25	2.98 ± 0.49
Myosin, heavy polypeptide 11, smooth muscle (NP_001019619; <i>D. rerio</i> )	1e-38	ATP binding GO:0005524, regulation of polarized epithelial cell differentiation GO:0030860	CB503691	-2.61 ± 0.45	-2.49 ± 0.25
Myosin, light polypeptide 7, regulatory (NP_571404; <i>D. rerio</i> )	5e-76	calcium ion binding GO:0005509	BU965885		-3.36 ± 0.24
<i>Cell adhesion</i>					
Cadherin 1, epithelial (NP_571895; <i>D. rerio</i> )	0	cell-cell adhesion GO:0016337, calcium ion binding GO:0005509	CB517027	2.81 ± 0.49	
Ependymin precursor (P38528; <i>Cyprinus carpio</i> )	1e-26	cell-matrix adhesion GO:0007160, calcium ion binding GO:0005509	CB509787 (4)	-3.92 ± 0.76	-3.51 ± 0.47
Junction plakoglobin (Q8SPJ1; <i>B. taurus</i> )	1e-16	protein binding GO:0005515	CB498556		2.13 ± 0.23
<i>Enzyme regulator activity</i>					
Tissue inhibitor of metalloproteinase 2 (AAU14867; <i>O. mykiss</i> )	8e-124	metalloendopeptidase inhibitor activity GO:0008191	CA054726	-2.31 ± 0.18	
<i>Binding</i>					
<b>C-type lectin 2 (BAC54021; <i>Anguilla japonica</i>)</b>	<b>2e-16</b>	<b>sugar binding GO:0005529</b>	<b>CB506151 (2)</b>	<b>2.61 ± 0.74</b>	
<i>Catalytic activity</i>					
<b>Natural killer cell enhancement factor (AAF71324; <i>O. mykiss</i>)</b>	<b>1e-110</b>	<b>antioxidant activity GO:0016209</b>	<b>CB505875</b>	<b>-2.40 ± 0.35</b>	<b>-2.54 ± 0.31</b>
<i>Miscellaneous</i>					
Anterior gradient-2-like protein 1 (ABB96968; <i>S. salar</i> )	2e-75		CB492865 (3)	2.37 ± 0.38	2.21 ± 0.13
Anterior gradient-2-like protein 2 (ABB96969; <i>S. salar</i> )	9e-77		CB504403 (4)	2.72 ± 0.50	2.14 ± 0.14
<b>C1q-like adipose specific protein (AAM73701; <i>Salvelinus fontinalis</i>)</b>	<b>1e-30</b>		<b>CK990940 (3)</b>	<b>14.08 ± 8.91</b>	<b>8.88 ± 0.77</b>
<b>C5a anaphylatoxin receptor (C5aR) gene CD88 (AY366354; <i>O. mykiss</i>)*</b>	<b>2e-19</b>		<b>CB517032</b>	<b>-3.26 ± 1.48</b>	
<b>Differentially regulated trout protein 1, DRTP-1 (AAG30030; <i>O. mykiss</i>)</b>	<b>3e-44</b>		<b>CB502879</b>		<b>2.82 ± 0.69</b>
Peroxisome proliferator-activated receptor $\beta$ 1B (AM229298; <i>S. salar</i> )*	3e-100		CK990580	-2.08 ± 0.23	
Tropomyosin 1 $\alpha$ (NP_957228; <i>D. rerio</i> )	2e-108		CB517835 (2)	-2.51 ± 0.42	-3.25 ± 0.19
Tumour protein D52 (NP_001038486; <i>D. rerio</i> )	2e-50		CA038353	-2.76 ± 0.99	-3.41 ± 0.26

Features shown were selected from non-redundant, annotated genes that were significantly different (Student's *t*-test,  $p < 0.05$ ) and  $\geq 2$ -fold differentially-regulated in either microarray study. For full gene lists and additional information see Supplementary Tables 1 and 2. Genes associated with the immune response in Atlantic salmon are shown in bold.

<sup>a</sup> Basic local alignment search tool (BLAST). The most significant (lowest E value<sup>†</sup>) BLASTX hit on contigs assembled from the expressed sequence tag (EST) by the Genome Research on Atlantic Salmon Project (GRASP). If a contig had no significant BLASTX hit ( $E < 1e^{-15}$ ), then the most significant BLASTN hit is shown and indicated with an asterisk. The UniProt/Swiss Prot, GenBank protein or nucleotide accession number is shown in parenthesis along with the species.

<sup>b</sup> Gene ontology derived from top BLAST hit and consistent with Gene Ontology nomenclature (GO: <http://www.geneontology.org/>).

<sup>c</sup> GenBank number of EST corresponding with GRASP microarray feature. When multiple features represented the same transcript a representative feature is provided and the number of additional features are shown in parenthesis.

<sup>d</sup> Fold change = mean relative expression  $\pm$  standard error.

<sup>e</sup> Genes were categorised by their parent gene ontology derived from the GO term.

While the *Sasa*-ZE transcript contains features of both functional and non-functional forms of MHC I, its immunological role is yet to be determined (Miller et al., 2006). Interestingly, multiple features with a best BLASTX hit to the non-functional MHC I transcript *Onmy*-UGA from rainbow trout were down-regulated. However, the identity of these features is ambiguous since the MHC IA/IB-independent (Lukacs et al., 2007) Atlantic salmon UGA locus (*Sasa*-UGA) has only been partially characterised (Miller et al., 2006) and the next best BLASTX hits for these features were encoded by the MHC I *Satr*-UBA\*0501 locus of brown trout (*Salmo trutta*) and *Sasa*-UBA (data not shown).

Atlantic salmon transcripts associated with professional antigen presenting cells (APCs) were down-regulated in AGD<sup>+</sup> lesions including complement 5a anaphylatoxin receptor (C5aR) (Boshra et al., 2006), IgM, a teleost-specific Ig heavy chain isotype (Ig $\tau$ ) (Hansen et al., 2005), the MHC II receptor complex subunits  $\alpha$  and  $\beta$  (*Sasa*-DAA and *Sasa*-DAB) (Hordvik et al., 1993) and MHC II invariant chain-like proteins (Iclp) similar to *Onmy*-Iclp 1/INVX, *Onmy*-Iclp 1/S25-7 and *Onmy*-Iclp 2/14-1 (Fujiki et al., 2003). Atlantic salmon possess similar transcripts to *Onmy*-Iclp 1/INVX (*Sasa*-Iclp 1) and *Onmy*-Iclp 2/14-1 (*Sasa*-Iclp 2) (Sakai et al., 2004). *Onmy*-Iclp 1/INVX and *Onmy*-Iclp 1/S25-7 are orthologues of the mammalian MHC II invariant chain (Ii) (Fujiki et al., 2003). A number of the Ii structural domains are missing in *Onmy*-Iclp 2/14-1 (*Sasa*-Iclp 2) and its functional role in the MHC II pathway is unclear (Fujiki et al., 2003). Genes associated with T cells were also down-regulated in AGD<sup>+</sup> lesions including the  $\alpha$  chain of the T cell receptor (Hordvik et al., 1996) and the cell-surface glycoprotein CD53 which, in mammals, regulates T cell maturation (Levy et al., 1998).

Key components of the MHC I and MHC II pathways discussed above are inducible by IFN cytokines (Boehm et al., 1997). Other IFN-inducible genes were down-regulated in AGD<sup>+</sup> lesions including an Atlantic salmon transcript similar to interferon regulatory factor-1 (IRF-1) from rainbow trout (Collet et al., 2003). IRF-1 is a transcriptional activator of interferon-induced molecules including key genes involved in the MHC I and MHC II antigen presentation pathways (Collet et al., 2003; Martin et al., 2007c). In addition, IFN- $\gamma$ -inducible genes allograft inflammatory factor 1 and guanylate-binding protein 1, both of which play a role in the regulation of inflammation (Deininger et al., 2002; Naschberger et al., 2004) were down-regulated in AGD<sup>+</sup> lesions.

A modest number of genes that regulate the early inflammatory response to tissue injury were dysregulated in AGD<sup>+</sup> lesions. Transcripts containing conserved domains homologous to complement component 1, subcomponent q (C1q) were dysregulated, including the down-regulation of C1q from the Norway rat (*Rattus norvegicus*) and up-regulation of C1q-like adipose-specific protein from brook trout (*Salvelinus fontinalis*). However, the Atlantic salmon C1q transcript represented on the microarray slide and was not differentially-regulated (data not shown). Differentially-regulated trout protein 1 (DRTP-1) was also up-regulated in AGD<sup>+</sup> lesions. Based on sequence similarity, rainbow trout DRTP-1 was suggested to be similar to CD59, a regulatory molecule that prevents damage to the host, mediated by complement membrane attack complex (Bayne et al., 2001; Boshra et al., 2006). A chemotactic cytokine (CC)-like protein with



similarity to the small inducible cytokine, SCYA102 from a rock-dwelling cichlid (*Melanochromis auratus*) (Kuroda et al., 2003) was up-regulated in AGD<sup>+</sup> lesions.

A transcript containing a carbohydrate recognition domain and similar to a galactose-binding C-type lectin from *Anguilla japonica* (C-type lectin 2) was up-regulated in AGD<sup>+</sup> lesions. In *A. japonica* C-type lectin 2 is highly expressed by mucous cells and was suggested to play a role in the immune response during transfer from seawater to freshwater (Mistry et al., 2001). Two C-type lectin receptors were differentially-regulated within AGD<sup>+</sup> lesions, immune-related, C-type lectin receptor A (CLRA) (Soanes et al., 2004) was up-regulated while C-type lectin receptor B was down-regulated.

#### **6.4.2.3 Genes associated with cell proliferation and re-modelling**

During gross examination, AGD-affected fish present (multi)focal patches on the gill, indicative of hyperplastic tissue. This alteration in tissue structure is evident at the histological level and now the transcriptional level, with several genes encoding structural proteins differentially-expressed in AGD<sup>+</sup> lesions. Matrix metalloproteinase 13 (MMP-13) which is capable of degrading type I collagen in rainbow trout (Saito et al., 2000) was up-regulated in AGD<sup>+</sup> lesions. This correlated with the simultaneous down-regulation of tissue inhibitor of metalloproteinase 2, an MMP and growth factor inhibitor (Stetler-Stevenson and Seo, 2005). In addition, the three subunits of type I collagen and secreted protein acidic and rich in cysteine (SPARC), critical for binding

collagen to the extracellular matrix (Brekken and Sage, 2000) were down-regulated in AGD<sup>+</sup> lesions. The predominant structural proteins up-regulated in AGD<sup>+</sup> lesions were type I and II cytokeratins. While in most mammals, cytokeratins are almost exclusively restricted to epithelial cells, in teleosts, cytokeratins are expressed by both epithelial and a variety of mesenchymal cells (Schaffeld and Marld, 2004). Type I and II “E” keratins constitute the keratin filaments expressed in the epidermis and other stratified epithelia, while type I and II “S” keratins are expressed in internal, simple epithelia and mesenchymal cells (Schaffeld et al., 2002a; Schaffeld et al., 2002b; Schaffeld and Marld, 2004). Genes similar to type I<sub>S</sub> keratins *Onmy*-K10 and *Onmy*-K18 and type II<sub>E</sub> keratins, *Onmy*-K1 and *Onmy*-K3 from rainbow trout were highly expressed in AGD<sup>+</sup> lesions. In mammals, the relationship between the proliferation of fibroblasts and keratinocytes is tightly regulated by the expression of the activator protein-1 transcription factor, composed of members of the Jun family of proteins (c-Jun, JunB and JunD) (Szabowski et al., 2000). JunB directly or indirectly suppresses the expression of cytokines that induce proliferation of keratinocytes in mesenchymal-epidermal interactions (Szabowski et al., 2000) and was up-regulated in AGD<sup>+</sup> lesions. Cell-to-cell adhesion molecules highly expressed in epithelial cells were also up-regulated in AGD<sup>+</sup> lesions, including epithelial cadherin (Mareel et al., 1993) and junction plakoglobin (Brakenhoff et al., 1995). In mice, claudin 23 is an integral membrane cell-adhesion protein which is a component of tight junctions that form the epidermal barrier (Gareus et al., 2007) and was down-regulated in AGD<sup>+</sup> lesions. Epidermal-type arachidonate lipoxygenase 3 (eLOX3) plays a pivotal role in the terminal differentiation of

keratinocytes (Furstenberger et al., 2007; Krieg et al., 2001; Yu et al., 2003) and was up-regulated in AGD<sup>+</sup> lesions. Conversely, the peroxisome proliferator-activated receptor subunit  $\beta$ , which causes a delay in keratinocyte differentiation (Tan et al., 2001) was down-regulated in AGD<sup>+</sup> lesions. In addition, Kruppel-like factor 11, which plays a central role in transforming growth factor- $\beta$ -induced c-myc repression and anti-proliferation of epithelial cells (Buck et al., 2006), was down-regulated in AGD<sup>+</sup> lesions.

#### **6.4.3 Verification of microarray results by qRT-PCR**

To verify the patterns of gene regulation observed across the microarray studies, six down-regulated and three up-regulated genes were examined by qRT-PCR using reverse transcribed aRNA from AGD<sup>+</sup> lesion and AGD<sup>-</sup> no lesion tissue as template (Fig. 6.4). Before qRT-PCR analyses commenced, due consideration was given to oligonucleotide design, particularly where genes have been putatively duplicated. Oligonucleotides were designed to amplify both putative type I and II  $\beta$ 2m molecules since both were predicted to form a functional MHC I complex (Magor et al., 2004; Shum et al., 1996). Oligonucleotides were designed to the Iclps similar to mammalian Ii, *Sasa*-Iclp 1 and *Onmy*- Iclp 1/INVX and S25-7 (Fujiki et al., 2003).

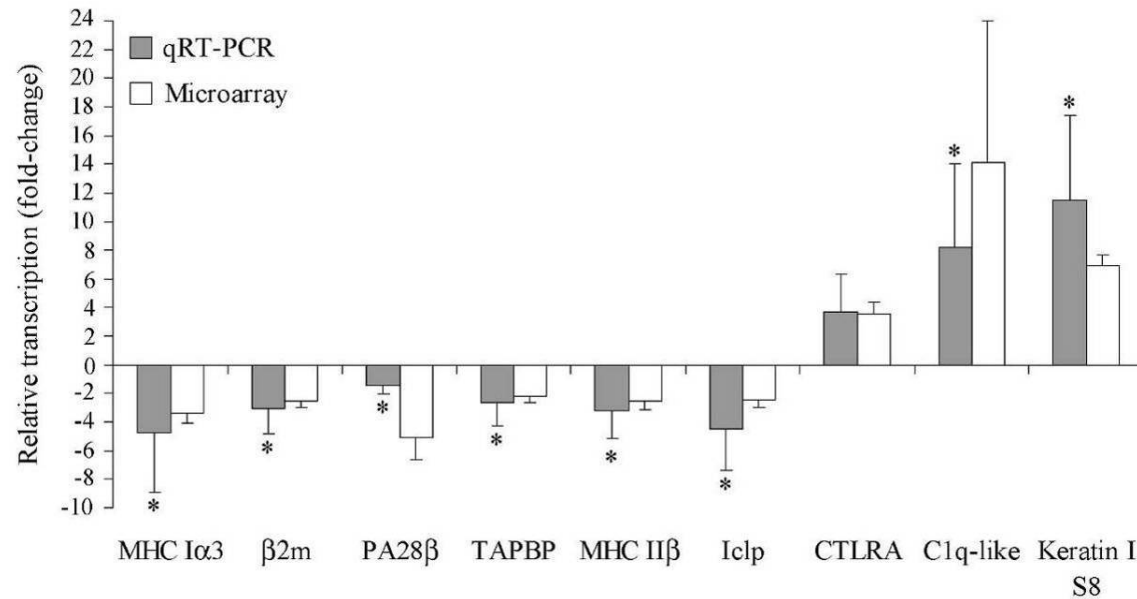


Figure 6.4. Quantitative real-time PCR (qRT-PCR) verification of microarray results. Microarray values are mean relative transcription (fold change)  $\pm$  standard error in AGD<sup>+</sup> lesion tissue relative to AGD<sup>-</sup> no lesion tissue (n = 6 fish) when significant (Student's *t*-test,  $p < 0.05$ ), otherwise the fold change in AGD<sup>+</sup> lesion is relative to AGD<sup>+</sup> no lesion tissue (n = 4 fish). The values shown are those of the feature represented in the microarray analysis gene list summary (Table 6.2). qRT-PCR verification of microarray analysis using aRNA is presented as mean fold change ( $\pm$  standard error) in AGD<sup>+</sup> lesion tissue (n = 5 fish) relative to AGD<sup>-</sup> no lesion tissue (n = 5 fish) at 36 days post-exposure to *Neoparamoeba perurans*. qRT-PCR values were normalised against ferritin, elongation factor 1 $\alpha$  and RNA polymerase II. Significant differences in gene expression are indicated with an asterisk ( $p < 0.05$ ). Genes examined were MHC I subunit  $\alpha$ 3 of the *Sasa*-UBA locus (MHC I $\alpha$ 3),  $\beta$ -2-microglobulin ( $\beta$ 2m), proteasome activator subunit 2  $\beta$  (PA28 $\beta$ ), TAP binding protein (TAPBP), MHC class II subunit  $\beta$ , MHC II invariant chain-like protein (Iclp), c-type lectin receptor A (CLRA), C1q-like adipose specific protein (C1q-like) and type I keratin S8 (Keratin I S8).

An Atlantic salmon transcript down-regulated in AGD<sup>+</sup> lesions was most similar to rainbow trout TAPBPb (*Onmy*-TAPBPb), a pseudogene which lacks the four terminal exons that encode the immunoglobulin superfamily C and V domains, transmembrane region and cytoplasmic tail (Landis et al., 2006). However, in Atlantic salmon, the deduced amino acid residues of MHC IA and MHC IB-encoded TAPBP loci (*Sasa*-TAPBP<sub>a</sub> and *Sasa*-TAPBP<sub>b</sub> respectively) shared 85.1% identity and 92.1% similarity and both retained the four terminal exons (Jørgensen et al., 2007a; Landis et al., 2006) (data not shown). Therefore qRT-PCR oligonucleotides were designed to amplify both *Sasa*-TAPBP<sub>a</sub> and *Sasa*-TAPBP<sub>b</sub>. The expression of each candidate gene was normalised to the three reference genes with the lowest coefficient of variation (% CV) and *M* scores, EF1 $\alpha$  (28.07%, 0.83), ferritin (36.08%, 0.89) and RPL2 (54.91%, 1.15). Overall, the patterns of gene regulation were consistent across the microarray and qRT-PCR platforms and only minor differences in the level of expression were observed (Fig. 6.4). qRT-PCR analysis of candidate genes yielded single amplicons when assessed by agarose gel electrophoresis and melt-curve analysis. Furthermore, the DNA sequence from an amplicon of each gene was homologous to the expected gene sequence.

#### **6.4.4 Time-course study of MHC I-related antigen processing and presentation gene expression**

Due to changes in gene regulation over the time-course study we focused on members of the MHC I pathway. The relative expression of mRNA from AGD<sup>+</sup> lesion, AGD<sup>+</sup> no lesion and AGD<sup>-</sup> no lesion tissue were assessed by qRT-PCR using the three reference genes described above, RPL2 (20.46% CV, 0.57 *M* score), EF1 $\alpha$  (25.91%, 0.66) and ferritin (29.97%, 0.67). In Atlantic salmon, some key genes

associated with antigen processing and presentation via the MHC I and MHC II pathways have been characterised and their structure and patterns of expression during disease are similar to their mammalian counterparts (Grimholt, 1997; Grimholt et al., 2002; Grimholt et al., 2000; Hordvik et al., 1996; Hordvik et al., 2004; Jørgensen et al., 2007b; Lukacs et al., 2007; Miller et al., 2006; Moore et al., 2005; Sakai et al., 2004; Yazawa et al., 2008). Other MHC I-related transcripts are yet to be described. Therefore, prior to analysis by qRT-PCR we confirmed the identity of these transcripts, including those described from rainbow trout. This was particularly important given nuances in MHC I A/B gene regions between rainbow trout and Atlantic salmon (Lukacs et al., 2007; Shiina et al., 2005). The incomplete (218 residues) deduced amino acid sequence of Atlantic salmon PA28 $\beta$  (*Sasa*-PA28 $\beta$ ) shared 88% identity and 94% similarity to the PA28 $\beta$  subunit of zebrafish (*Danio rerio*) and contained the conserved KEKE motif of the PA28 family of proteins (Murray et al., 2000; Realini et al., 1994). The deduced amino acid sequence of Atlantic salmon IRF-1 (*Sasa*-IRF-1) contained the IRF family signature of six tryptophan residue repeats in the first 77 N-terminal amino acids, spaced by 7 – 18 amino acids: NH<sub>2</sub>-X<sub>10</sub>-W-X<sub>14</sub>-W-X<sub>11</sub>-W-X<sub>7</sub>-W-X<sub>11</sub>-W-X<sub>18</sub>-W (Collet et al., 2003; Harada et al., 1989). In a pair-wise comparison with rainbow trout IRF-1/2 sequences (Collet et al., 2003), *Sasa*-IRF-1 shared 72% identity and 76% similarity to IRF-1 but only 32% identity and 49% similarity to IRF-2 suggesting that *Sasa*-IRF-1 was indeed IRF-1. The deduced amino acids from two dysregulated Atlantic salmon  $\beta$ 2m transcripts corresponded to rainbow trout type I  $\beta$ 2m (99% identity and similarity) and type II  $\beta$ 2m (96% identity and 99% similarity). Atlantic salmon type I and II  $\beta$ 2m only differed in a cluster of five residues between the positions 13–16 of the mature peptide, with type I encoding NFGDK and type II encoding EHGKD (data not shown). Atlantic salmon IFN- $\gamma$  (GenBank accession no. [AJ841811](#)) shared 89%

identity and 93% similarity to rainbow trout and contained the proposed IFN- $\gamma$  family motif at amino acid residues 138 to 147: Q-X-[KQ]-A-X<sub>2</sub>-E-[LF]-X<sub>2</sub> (Zou et al., 2005).

In general, all candidate genes associated with MHC I antigen presentation were down-regulated over the time course study and this differential-regulation was primarily restricted to AGD-gill lesions (Fig. 6.5). Across candidate genes, the patterns and magnitude of gene expression obtained using template derived from mRNA and aRNA were consistent when AGD<sup>+</sup> lesion tissue was compared to AGD<sup>-</sup> no lesion tissue at 36 DPE to *N. perurans* [mean  $\pm$  standard error; MHC I $\alpha$ 3 (-5.8  $\pm$  3.2 by mRNA vs. -4.8  $\pm$  4.1 by aRNA),  $\beta$ 2m (-3.2  $\pm$  1.6 by mRNA vs. -3.1  $\pm$  1.8 by aRNA) and TAPBP (-2.5  $\pm$  1.4 by mRNA vs. -2.6  $\pm$  1.6 by aRNA)] (Fig. 6.4 and Fig. 6.5). When the less discernable gill lesions taken from fish with low AGD severity at 12 DPE to *N. perurans* were assessed in relation to non-lesion tissue, there were no significant differences in the expression of any candidate genes ( $p > 0.05$ ). At 25 and 36 DPE to *N. perurans*, the primary components of MHC class I complex (MHC I and  $\beta$ 2m) were both significantly down-regulated ( $p < 0.05$ ) in AGD<sup>+</sup> lesion tissue compared to both AGD<sup>-</sup> no lesion tissue (MHC I $\alpha$ 3 -4.6 to -5.8-fold change,  $\beta$ 2m -3.0 to -3.2-fold change) and AGD<sup>+</sup> no lesion tissue (MHC I $\alpha$ 3 -3.0 to -4.9-fold change,  $\beta$ 2m -2.4 to -3.2-fold change), consistent with microarray analyses. A similar pattern was observed for TAPBP, when expression in AGD<sup>+</sup> lesions was compared to AGD<sup>-</sup> no lesion tissue (-2.5 to -3.7-fold change) and AGD<sup>+</sup> no lesion tissue (-2.4-fold change). IRF-1 was significantly ( $p < 0.05$ ) down-regulated at 25 (-3.2-fold change) and 36 (-1.7-fold change) DPE to *N. perurans* in AGD<sup>+</sup> lesion tissue in relation to AGD<sup>-</sup> no lesion tissue but only significantly down-regulated at 36 DPE to *N.*

*perurans* (-2.1-fold change) in AGD<sup>+</sup> lesion tissue in relation to AGD<sup>+</sup> no lesion tissue.

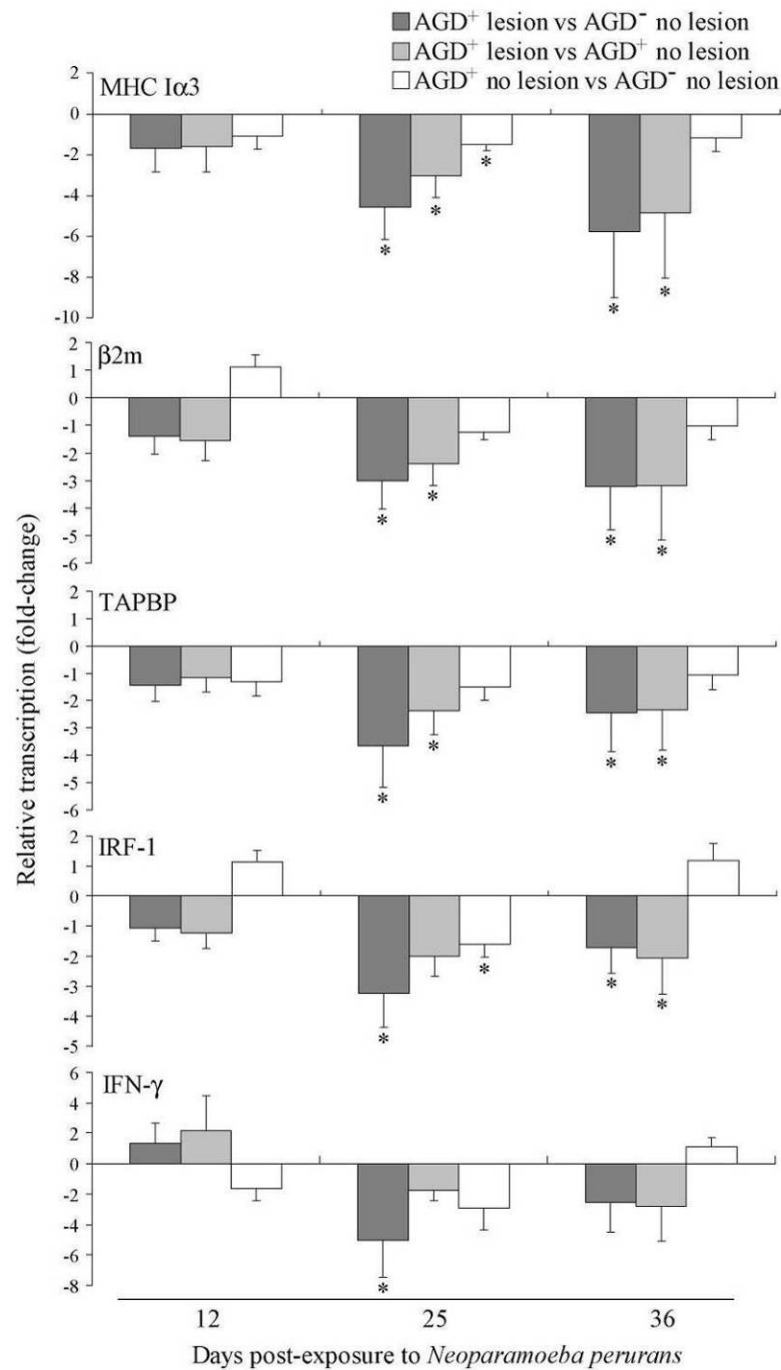


Figure 6.5. Relative transcription (fold change) of interferon- $\gamma$  (IFN- $\gamma$ ) and IFN-inducible genes associate with the MHC class I pathway by quantitative real-time PCR (qRT-PCR) analysis of mRNA. Data are presented as mean fold change ( $\pm$  standard error) in each gene when samples of AGD<sup>+</sup> lesion tissue (n = 5 fish), AGD<sup>-</sup> non-lesion tissue (n = 5 fish) and AGD<sup>+</sup> non-lesion tissue (n = 5 fish) were compared to each other at 12, 25 and 36 days post-exposure to *Neoparamoeba perurans*. qRT-PCR values were normalised against ferritin, elongation factor 1 $\alpha$  and RNA polymerase II. Significant differences in gene regulation are indicated with an



asterisk ( $p < 0.05$ ). Genes examined were MHC I subunit  $\alpha 3$  of the *Sasa*-UBA locus (MHC I $\alpha 3$ ),  $\beta$ -2-microglobulin ( $\beta 2m$ ), TAP binding protein (TAPBP), interferon regulatory factor I (IRF-1) and IFN- $\gamma$ . IFN- $\gamma$  was not represented on the microarray slide but its importance in regulating key components of the MHC I and MHC II pathways (Boehm et al., 1997) justified its analysis. IFN- $\gamma$  is the most potent cytokine that induces MHC I expression as well as other genes involved in antigen processing and presentation and was down-regulated (-5.1-fold change) in AGD<sup>+</sup> lesions in relation to AGD<sup>-</sup> no lesion tissue at 25 DPE to *N. perurans* ( $p < 0.05$ ). Variable expression of IFN- $\gamma$  precluded the identification of statistically significant differences at the other time points. Again, all qRT-PCR assays yielded single amplicons when assessed by agarose gel electrophoresis and melt curve analysis. Furthermore, the DNA sequence from an amplicon of each gene was homologous to the expected gene sequence.

## 6.5 Discussion

Some sea cage-cultured marine fishes are highly susceptible to AGD and if untreated, AGD epizootics lead to devastating losses of stock (Munday et al., 2001). To date there is little, if any, definitive evidence that AGD-affected fish have the capacity to develop innate (Bridle et al., 2006a; Bridle et al., 2006b; Morrison et al., 2007) or acquired (Akhlaghi et al., 1996; Findlay and Munday, 1998; Gross et al., 2006; Gross, 2007; Gross et al., 2004b; Morrison et al., 2006a; Vincent et al., 2006) immunity to the condition. Therefore, in pursuit of the mechanism(s) that may undermine the provision of host resistance, we used a salmonid microarray as a platform to examine the transcriptome response in AGD-affected gill tissue. Secondly, experiments were designed to address the hypothesis that dysregulation of genes in the gills of AGD-affected fish is restricted to the lesions themselves. Overall, microarray and qRT-PCR analyses demonstrated that the majority of

differential gene expression was restricted to AGD<sup>+</sup> lesions. When dysregulated genes were assigned to functional gene ontology categories, the data were punctuated by two groups of transcripts: (1) Those associated with changes in tissue architecture and (2) Those associated with the immune response. Of the latter, AGD-affected fish display no evidence of a coordinated innate or adaptive immune response. Rather, in AGD<sup>+</sup> lesions, the response featured a down-regulation of IFN- $\gamma$  and multiple IFN-inducible transcripts, in particular those involved in processing and presentation of antigens via the MHC I and MHC II pathways.

Quite clearly there is a profound change in the gill tissue architecture during AGD (Fig. 6.1D) and several significantly modulated transcripts described here reflect this change. Of these, some transcripts may be genuinely dysregulated, while others may retain a “normal” copy number but appear differentially expressed on the basis of structural change. Significant dysregulation of transcripts encoding several intermediate filaments (cytokeratins) suggests that hyperproliferative epithelial cells manifest AGD lesions, consistent with previous studies (Dyková et al., 1995; Kent et al., 1988; Roubal et al., 1989). In fish, unlike mammals, keratin filaments are expressed by epithelial and mesenchymal cells (Schaffeld and Marld, 2004) and both these lineages may be involved in AGD lesions. However, up-regulation of other epithelial-specific transcripts (e.g. e-cadherin, junction plakoglobins, eLOX3) concomitant with down-regulation in mesenchymal cell-derived collagen (I $\alpha$  1, 2 & 3) indicates that on balance, epithelial cells are most likely the predominant cell phenotype (Dyková et al., 1995; Kent et al., 1988; Roubal et al., 1989).

Another lineage of cells described in cases of AGD are eosinophils, which are claimed to be the primary infiltrating cells in AGD lesions (Lovy et al., 2007). However, when transcripts dysregulated in AGD<sup>+</sup> lesions were compared with highly up-regulated transcripts characteristic of mammalian eosinophils (Blanchard et al., 2006; Nakajima et al., 2001) there was no evidence of eosinophilia at the transcriptional level. Of the 71 genes that were up-regulated in mammalian eosinophils, 20 were represented on the GRASP microarray, yet only one was up-regulated (S100 calcium binding protein). These seemingly contradictory findings suggest that the so-called eosinophils were misidentified (since they were described on the basis of morphology alone), they are equally abundant in AGD lesions as in normal tissue or they possess a transcriptome distinct from that of mammalian eosinophils.

Others transcripts, while not directly involved in these structural changes, may be indirectly affected. For example, differential expression of the haemoglobin  $\alpha$  and  $\beta$  subunits and Na<sup>+</sup>/K<sup>+</sup> ATPase subunits may simply be an artefact manifest by the excision of AGD lesions. Similarly, down-regulation of the MHC II antigen processing and presentation pathway could be mediated by differences in tissue type. In other words, there are fewer cells expressing MHC II in AGD lesions than in unaffected tissue. It is equally plausible, given that there are MHC II  $\beta$ <sup>+</sup> cells present in AGD<sup>+</sup> lesions (Morrison et al., 2006b), that MHC II is legitimately down-regulated. In the absence of data to resolve this issue, we chose to focus our attention on the MHC I antigen processing and presentation pathway. Salmonid MHC I molecules are ubiquitously expressed in a wide variety of tissues including epithelial, endothelial, lymphoid tissues and leucocytes (Aoyagi et al., 2002; Dijkstra et al.,

2003a; Fischer et al., 2005; Hansen and La Patra, 2002; Hansen et al., 1999; Jørgensen et al., 2007b; Miller et al., 2006). MHC I-related genes are also expressed in a similar manner including  $\beta 2m$  (Jørgensen et al., 2006b; Magor et al., 2004), TAP (Hansen et al., 1999) and TAPBP (Landis et al., 2006). Furthermore, MHC I is highly expressed in healthy rainbow trout gill epithelial cells (Dijkstra et al., 2003a; Fischer et al., 2005), indicating that a down-regulation in expression of MHC I pathway in AGD<sup>+</sup> lesions, unlike the MHC II pathway, is not as easily attributable to a shift in cell type.

The coordinated down-regulation of multiple MHC I-related genes in AGD<sup>+</sup> lesions suggests that the activity of one or more DNA-binding transcription factors common to all transcripts is suppressed. In mammals, the transcriptional regulation of MHC I is mediated by TNF- $\alpha$  through NF- $\kappa$ B (Drew et al., 1995; Johnson and Pober, 1994) and/or IFN via IRF (Boehm et al., 1997; Goodbourn et al., 2000). Similarly, in fish, IFN- $\gamma$  induces the expression of IRF-1, MHC I and MHC I-related transcripts such as TAP1, LMP7 (PSMB8), LMP2 (PSMB9) and tapasin (TAPBP) in macrophages (Martin et al., 2007b). Despite AGD lesion-restricted up-regulation of IL-1 $\beta$  mRNA, TNF- $\alpha$  transcripts are not typically modulated in AGD<sup>+</sup> lesions (Morrison et al., 2009 manuscript in preparation; Morrison et al., 2007) therefore no further consideration to TNF- $\alpha$  and NF- $\kappa$ B is given in this discussion. IFN-induced expression of mammalian MHC I is mediated by either type I (IFN $\alpha/\beta$ ) or more strongly by type II (IFN- $\gamma$ ) interferons through IRF-1 (Sims et al., 1993). Atlantic salmon type I IFN- $\alpha$  (Robertsen et al., 2003), whilst represented on the microarray chip, was not differentially-regulated in AGD-affected tissue. When examined, expression of IFN- $\gamma$  was highly variable as previously observed (Morrison et al., 2007) but significantly

down-regulated in coordination with IFN- $\gamma$ -inducible genes at 25 DPE to *N. perurans* in AGD<sup>+</sup> lesions. Down-regulation of IFN- $\gamma$  did not occur at 36 DPE, despite significant down-regulation of IRF-1, MHC I and MHC I-related genes. Transcriptional modulation of IRF-2, which binds the same gene promoter-based motifs as IRF-1 and acts as a transcriptional repressor of IRF-1-inducible genes (Taniguchi et al., 2001) may account for this discrepancy. However IRF-2 was not differentially-regulated in AGD lesions (data not shown). Thus, down-regulation of MHC I and related transcripts may be influenced solely by transcriptional suppression of IRF-1 and regulation of IRF-1 may be influenced by IFN- $\gamma$  but is also likely to be driven by factors other than IFN- $\gamma$ .

On face value, the coordinated down-regulation of the MHC I antigen processing and presentation pathway appears to be a vehicle for parasite-mediated immune evasion. While this hypothesis is logical given the range of sophisticated methods of immune evasion used by parasites (Sacks and Sher, 2002) other, equally plausible explanations may be valid. In the classical MHC I antigen processing pathway, antigenic peptides are derived from either cytosolic proteins that are the result of new synthesis or those that enter through the cell membrane via an endosome or pinosome (Townsend and Bodmer, 1989). The MHC I complex is displayed on the cell surface where it is inspected by cytotoxic CD8<sup>+</sup> T cells which then proceed to kill offending cells. The classical MHC I pathway is therefore the major defence against endogenously derived antigens, primarily viruses, intracellular bacteria and tumours. Viruses are the most well known modulator of the MHC I pathway by negative cytokine regulation or disruption of antigenic peptide processing and presentation (Hewitt, 2003; Ploegh, 1998). Previously, we considered the “Trojan

horse” concept for *N. perurans* and AGD, hypothesising that *N. perurans* was merely a vector for viral infection. However, *N. perurans* lysates generated by freeze-thaw or physical disruption failed to elicit a cytopathic effect on either the salmonid (CHSE) or carp (EPC) cell lines (Nowak et al., 2004). In addition, if viral co-infection was to occur, then freshwater bathing routinely used by commercial Atlantic salmon growers to successfully treat AGD (Munday et al., 2001) would not be efficacious. Perhaps more likely, is that the restricted expression of MHC I-related genes can be attributed to the state of cell differentiation, as described in mammalian cells (Fontana et al., 1987; Israel et al., 1989; Miyazaki et al., 1986). Equally, the selection of a cell lineage with an MHC I low expression phenotype in AGD<sup>+</sup> lesions may occur. Whatever the case, the functional outcomes of our observations are ultimately most important. Down-regulation of the mammalian antigen processing and presentation machinery reduces the sensitivity of cells to cytotoxic T cell-mediated lysis (Bukowski and Welsh, 1985; Pereira et al., 1995). However, this is of no relevance in the AGD context unless down-regulation of MHC I is a mechanism to suppress the ability to cross-present extracellular antigen to CD8<sup>+</sup> T cells. This issue clearly requires further investigation, particularly since there is no evidence of cross-presentation in teleost fish.

In summary, we present evidence that shows differential expression of transcripts in the gills of AGD-affected fish is mostly restricted to lesions. The lesion-restricted down-regulation of several genes involved in antigen processing and presentation, together with an up-regulation of DRTP (CD59), is characteristic of sites of immune privilege in mammals (Hong and Van Kaer, 1999). Whether or not this inhibits the development of resistance to AGD is unknown and further studies are required.

Initially, our ongoing research will focus on the effects of rIFN- $\gamma$  on AGD lesion-derived cells *in vitro* and AGD-affected fish *in vivo*.

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**7 STIMULATION OF AMOEBCIC GILL DISEASE-AFFECTED  
ATLANTIC SALMON (*SALMO SALAR*) GILLS WITH INTERFERON- $\gamma$   
FAILS TO RESTORE NORMAL EXPRESSION OF MHC CLASS I/ $\beta$ 2m  
mRNAs**

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Manuscript in preparation:

Young N. D., Safavi-Hemami H., Nowak B. F. and Morrison R. N. (2009)  
Stimulation of amoebic gill disease-affected Atlantic salmon (*Salmo salar*)  
gills with interferon- $\gamma$  fails to restore normal expression of MHC class I/ $\beta$ 2m  
mRNAs.



## 7.1 Abstract

Atlantic salmon (*Salmo salar*) are highly susceptible to amoebic gill disease (AGD), a potentially fatal condition. An attribute of AGD lesions is the down-regulation in the MHC class I (MHC I) antigen processing and presentation pathway, an important component of the host defence from pathogens and maintenance of cell homeostasis. Since the disruption of the MHC I pathway was shown to be associated with a down-regulation of interferon regulatory factor 1 (IRF-1), a transcriptional activator of MHC I-associated genes, the changes in the AGD lesions may be under epigenetic control and therefore reversible. IFN- $\gamma$  activates IRF-1-mediated induction of the MHC I pathway and its expression patterns over the course of an infection were ambiguous. Therefore in this study, we investigated whether IFN- $\gamma$  treatment could restore the expression of the MHC I antigen presentation pathway in AGD lesions. A new isoform of Atlantic salmon IFN- $\gamma$  was cloned, sequenced and expressed in *E. coli*. Initially, the recombinant IFN- $\gamma$  (rIFN- $\gamma$ ) was shown to be biologically active by virtue of its capacity to induce transcription of MHC I-associated genes in head kidney leucocytes. Stimulation of AGD lesions with rIFN- $\gamma$  *ex vivo* failed to restore normal expression of MHC I/ $\beta$ 2m mRNAs despite the induction of  $\gamma$ -IP10 and IRF-1. Co-induction of IRF-8, a known suppressor of IRF-1-mediated transcription indicated that the MHC I/ $\beta$ 2m quiescence was not the result of aberrant IFN- $\gamma$  receptor expression but possibly antagonism at the intracellular signalling level. However, upon further examination, IFN- $\gamma$  failed to induce IRF-1 or MHC I-associated genes in the gills of healthy fish, suggesting that the transcriptional activation of the MHC I antigen presentation pathway in the gills of fish may be independent of IFN- $\gamma$  or steadfastly stable following stimulation with cytokines known to induce MHC I in mammals.

## 7.2 Introduction

Amoebic gill disease (AGD) is an ectoparasitic condition affecting some marine fish (Dyková et al., 1995; Kent et al., 1988; Munday et al., 1990; Rodger and McArdle, 1996; Roubal et al., 1989). AGD can be fatal if left untreated and is a significant cause of mortality in marine fish reared under farm conditions (Dyková et al., 1995; Kent et al., 1988; Munday et al., 1990; Rodger and McArdle, 1996; Young et al., 2008c). Gross signs of AGD are restricted to the gills and present as multi-focal lesions, visible as pale gill tissues (Munday et al., 1990; Rodger and McArdle, 1996). AGD lesions develop following the association of *Neoparamoeba perurans* trophozoites with gill tissue (Young et al., 2007) and are characterised primarily by hyperplasia of epithelial-like cells that results in extensive secondary lamellar fusion (Dyková et al., 1995; Kent et al., 1988; Munday et al., 1990; Rodger and McArdle, 1996; Roubal et al., 1989) and progressive loss of normal gill structure (Adams and Nowak, 2003).

The outer epithelial layer of a fish constitute the first line of defence against ectoparasitism, providing the host with a layer of physical protection as well as tissue capable of secreting an array of compounds that initiate and modulate the immune response (Gonzalez et al., 2007a; Gonzalez et al., 2007b; Lindenstrom et al., 2003; Lindenstrom et al., 2004; Singh et al., 2004a; Singh et al., 2004b). Like most vertebrates, fish employ a repertoire of host defence mechanisms against ectoparasites that work cooperatively to develop a cell-mediated inflammatory response (Buchmann et al., 2001; Chin and Woo, 2005; Cross and Matthews, 1993; Hines and Spira, 1974a) and acquired resistance to infection (Bakke et al., 1991; Chin et al., 2004; Clark et al., 1987; Hines and Spira, 1974b; Lindenstrom and

Buchmann, 2000; Singh and Buchmann, 2001). Interestingly, other than hyperplasia of epithelial-like cells, there is only a modest cellular response to *N. perurans* infection in Atlantic salmon (*Salmo salar*). Some leucocytes migrate to the central venous sinus, adjacent to AGD lesions but seldom are they seen within the lesions themselves (Adams and Nowak, 2001; Adams and Nowak, 2003). Furthermore, there is little, if any, definitive evidence that AGD-affected fish have the capacity to develop innate (Bridle et al., 2006a; Bridle et al., 2006b; Morrison et al., 2007) or acquired (Akhlaghi et al., 1996; Findlay and Munday, 1998; Gross et al., 2006; Gross, 2007; Gross et al., 2004b; Morrison et al., 2006a; Vincent et al., 2009; Vincent et al., 2006) resistance that minimises the impact of ectoparasitosis by *N. perurans*.

At the molecular level, transcriptome-wide profiling of AGD-affected Atlantic salmon gills provided further evidence that fish fail to mount an innate or adaptive immune response to ectoparasitosis by *N. perurans* (Morrison et al., 2006a; Young et al., 2008a). Instead, the transcriptome of cells in AGD lesions is characterised by a coordinated down-regulation in the mRNA expression of major histocompatibility complex class I (MHC I) and class II (MHC II) antigen processing and presentation pathways (Young et al., 2008a). In salmonids, the expression of MHC II molecules is generally restricted to professional antigen presenting cells (Koppang et al., 1998), thus the down-regulation in the expression of MHC II molecules may well have been an artefact of the differences in the cell types that constitute an epithelial cell-rich AGD lesion (Young et al., 2008a). In contrast, salmonid MHC I molecules are ubiquitously expressed in a wide variety of tissues (Aoyagi et al., 2002; Dijkstra et al., 2003a; Fischer et al., 2005; Hansen and La Patra, 2002; Hansen et al., 1999;

Jørgensen et al., 2007b; Miller et al., 2006), including gill epithelial cells (Dijkstra et al., 2003a; Fischer et al., 2005; Scharsack et al., 2007). Therefore a down-regulation in expression of MHC I pathway in AGD lesions is not as easily attributable to a shift in cell type and warranted further investigation.

The MHC I antigen processing and presentation pathway is a central component of adaptive cellular response to microbial pathogens and recognition of altered self cells (e.g. virus-infected cells and tumour cells). Traditionally MHC class I molecules present endogenously-derived antigens to the T cell receptor (TCR)  $\alpha\beta$ /CD8 complex of CD8<sup>+</sup> T cells (Sprent and Schaefer, 1985). The expression of MHC I molecules on the surface of healthy cells is also important for avoiding natural killer (NK) cell-mediated cell lysis (Johansson et al., 2005; Makrigrannis and Anderson, 2003). The transcriptional regulation of MHC I molecules is modulated by tumour necrosis factor-  $\alpha$  (TNF- $\alpha$ ) through NF- $\kappa$ B signalling (Drew et al., 1995; Johnson and Pober, 1994), interferon regulatory factor (IRF) (Hobart et al., 1997; Paun and Pitha, 2007) or interferon (IFN) signalling (Boehm et al., 1997; Goodbourn et al., 2000). Within AGD lesions, the down-regulation of MHC I antigen processing-related genes was associated with a down-regulation in IRF-1, a transcriptional activator of the MHC I antigen processing and presentation pathway in mammals (Hobart et al., 1997; Paun and Pitha, 2007). In addition, IFN- $\gamma$ , a potent modulator of IRF-1 and other MHC I genes in mammals (Hobart et al., 1997; Paun and Pitha, 2007), was variably expressed but down-regulated at 25 days post-exposure to *N. perurans* in AGD lesions (Young et al., 2008a). Thus the observed changes within AGD lesions appear to be epigenetic in origin and IFN- $\gamma$  may be acting as a central regulator of MHC I antigen processing and presentation.

The major focus of this study was to determine whether the down-regulation in the MHC I antigen processing and presentation pathway in AGD lesions was functionally relevant by restoring "normal" MHC I expression *in vivo*. As a first step in achieving this objective, this study establishes whether IFN- $\gamma$  is capable of restoring "normal" MHC I expression *ex vivo*. Dissociated AGD lesion-derived cells were stimulated with an Atlantic salmon recombinant IFN- $\gamma$  (rIFN- $\gamma$ ) *ex vivo* and the transcriptional responses of IRF-1, MHC I and  $\beta$ 2m were compared to dissociated cells derived from normal gill tissues. IFN- $\gamma$  signalling failed to induce expression of MHC class I/ $\beta$ 2m mRNAs in AGD lesions and normal gill tissues suggesting that the type II cytokine IFN- $\gamma$  may not be a critical regulator of MHC I antigen presentation in the gills of Atlantic salmon.

### **7.3 Materials and methods**

#### **7.3.1 PCR amplification of an Atlantic salmon interferon- $\gamma$ transcript**

Gill tissues used to amplify the Atlantic salmon IFN- $\gamma$  transcript were obtained from experiments previously described (Young et al., 2008a). Briefly, gill filaments were excised from healthy salt-water acclimated Atlantic salmon. Total RNA was purified using an RNeasy Mini kit (Qiagen, Doncaster, Victoria, Australia), a Dounce homogeniser (Wheaton Scientific, Millville, NJ, USA) and QIAshredders (Qiagen). All total RNA was DNase I-treated using TurboDNA-free (Ambion, Scoresby, Victoria, Australia). RNA extraction and DNase I treatment were performed according to the manufacturer's instructions. Total RNA concentrations were determined using a spectrophotometer and RNA integrity was verified by agarose gel

electrophoresis. For rapid amplification of cDNA ends (RACE), 5' and 3' universal primer-adapted cDNA was prepared from 900 ng of total RNA using the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, Mountain View, California, USA) as per manufacturer's instructions. RACE was performed using primary and nested universal oligonucleotides and three gene-specific oligonucleotides (Table 7.1) designed using available Atlantic salmon IFN- $\gamma$  transcripts. PCR was used to generate 5' and 3' RACE cDNA. Primary PCR reactions were performed in volumes of 50  $\mu$ L containing 2.5  $\mu$ L of 1:10 diluted cDNA, 0.625 U of Sahara DNA Polymerase (Bioline, Alexandria, NSW, Australia) which possesses 5'-3' polymerase activity and 3'-5' proofreading activity, 10 $\times$  Sahara DNA Polymerase reaction buffer (Bioline), 200  $\mu$ M of each deoxynucleotide triphosphate (dNTP, dATP, dCTP, dGTP and dTTP), 1.5 mM of MgCl<sub>2</sub>, 5  $\mu$ L of 10 $\times$  universal primer A mix (Clontech) and 0.2  $\mu$ M of a gene-specific oligonucleotide (Table 7.1). PCR cycle conditions were 5 cycles of 94 °C for 30 s and 72 °C for 3 min; 5 cycles of 94 °C, 70 °C for 30 s and 72 °C for 3 min; then 25 cycles of 94 °C, 68 °C for 30 s and 72 °C for 3 min. If the PCR reaction did not produce a visible PCR amplicon, a nested PCR reaction was performed. The nested PCR reactions were performed as described above except 5  $\mu$ L of the primary PCR reaction was used as DNA template and oligonucleotides were replaced with 1  $\mu$ L of nested universal primer A (Clontech) and 0.2  $\mu$ M of the nested gene-specific oligonucleotide (Table 7.1). Nested PCR cycle conditions were 25 cycles of 94 °C, 68 °C for 30 s and 72 °C for 3 min. All PCR amplicons were analysed by gel electrophoresis then nucleotide sequencing. The methods used to ligate PCR products into a plasmid vector, transformation of *Escherichia coli*, colony

Table 7.1. Sequences of oligonucleotides used in this study

Gene	Oligonucleotide	Sequence 5'-3'	Amplicon (bp)	Reference
Interferon- $\gamma$ (IFN- $\gamma$ )	3' RACE PCR	F-GGGAAGGCTCTGTCCGAGTTCATTACC		DY738740 <sup>d</sup>
	5' RACE PCR	R-AGAGCCTTCCCCTGGACTGTGGTG		DY738740 <sup>d</sup>
	5' RACE PCR nested	R-TGGTCCACCGTCTGGTTCAGCATC		DY738740 <sup>d</sup>
	Mature peptide	F-GCTCAGTACACATCAATTAAC, R- CATGATGTGTGATTTGAGC	468	DY738740 <sup>d</sup>
	qRT-PCR <sup>c</sup>	F-GGCTCTGTCCGAGTTCATTACC, R-GGGCTTGCCGTCTCTTCC	98	Young et al. 2008a
MHC class I (MHC I) <sup>a</sup>	qRT-PCR	F-CTGCATTGAGTGGCTGAAGA, R-GGTGATCTTGCCGTCTTTC	176	Jorgensen et al. 2006
$\beta$ -2-microglobulin ( $\beta$ 2m)	qRT-PCR	F-TCCCAGACGCCAAGCAG, R-TGTAGGTCTTCAGATTCTTCAGG	138	Young et al. 2008a
IFN- $\gamma$ -inducible CXCL10-like protein ( $\gamma$ IP-10)	qRT-PCR	F-ACATCAACAGTCCTCATCAGC, R- TCCGTTCTTCAGAGTGACAATG	201	DR696064 <sup>d</sup>
Interferon regulatory factor 1 (IRF-1)	qRT-PCR	F-ACAGTCAAGAGAGCCAATG, R-CCAGTAGTCGGTGTAAGAG	119	Young et al. 2008a
Interferon regulatory factor 2 (IRF-2)	qRT-PCR	F-CTTCATCAGAGCAGTCACAGTC, R-TCCTCATTCTCCGTGGTCAC	98	EF067842 <sup>d</sup>
Interferon regulatory factor 8 (IRF-8)	qRT-PCR	F-CCCATTGTGACCCTGTGTTTCG, R-ACTGGAGTGACCTGGACGATG	86	BM414289 <sup>d</sup>
Elongation factor 1 $\alpha$ (EF1 $\alpha$ ) <sup>b</sup>	qRT-PCR	F-TGATTGTGCTGTGCTTATCG, R-AACGCTTCTGGCTGTAGG	173	Young et al. 2008a
RNA polymerase II (RPL2) <sup>b</sup>	qRT-PCR	F-TAACGCCTGCCTCTTCACGTTGA, R-ATGAGGGACCTGTAGCCAGCAA	112	Jorgensen et al. 2006

<sup>a</sup> Major histocompatibility complex class I receptor, primers designed within the conserved  $\alpha$ 3-subunit of the *Sasa*-UBA locus

<sup>b</sup> Reference genes used to normalise relative expression values

<sup>c</sup> Quantitative reverse-transcription PCR (qRT-PCR)

<sup>d</sup> GenBank (<http://www.ncbi.nlm.nih.gov/>) nucleotide accession number

selection and plasmid purification were as previously described (Young et al., 2007). Sequencing reactions were initiated using an M13 forward (17-mer, Promega, Annandale, NSW, Australia) or an M13 reverse oligonucleotide (22-mer, Promega) and analysed on an ABI 3730xl DNA analyser (Applied Biosystems (ABI), Scoresby, Victoria, Australia) according to manufacturer's instructions.

### **7.3.2 Expression and purification of recombinant IFN- $\gamma$**

The putative mature peptide of Atlantic salmon IFN- $\gamma$  isoform 2 (IFN- $\gamma$ 2) was predicted using SignalP software (Fig. 7.1) (Emanuelsson et al., 2007). Initially the mature IFN- $\gamma$ 2 peptide was PCR-amplified using oligonucleotides that were designed so that only the mature peptide was amplified including 156 aa residues. The 5' end of the PCR product represents the first codon of the mature IFN- $\gamma$ 2 peptide and the stop codon was omitted from the 3' end of the PCR product (Table 7.1). PCR conditions were similar to the primary PCR conditions described for RACE PCR, except PCR cycling was 94°C for 8 min, 35 cycles of 94°C for 30 sec, 53°C for 30 sec and 72°C for 45 sec with a final extension step of 5 min at 72°C. An expression construct encoding a 6 $\times$  N-terminally His-tagged IFN- $\gamma$ 2 mature peptide was created by ligating the IFN- $\gamma$ 2 PCR amplicon into the pQE30 UA vector using the QIAexpress UA cloning kit (Qiagen) as per manufacturer's instructions. The construct was then transformed into the Rosetta strain of *E. coli* containing the pRARE plasmid (Novagen, Kilsyth, Victoria, Australia) as per manufacturer's instructions. Plasmid DNA was extracted from a clone containing the expression construct using QIAprep miniprep plasmid purification kit (Qiagen) and verified by sequencing both strands of DNA.



For purification of Atlantic salmon rIFN- $\gamma$ 2, a clone containing the expression construct was used to inoculate 5 mL of Luria broth (LB; Sigma) medium containing ampicillin (100  $\mu$ g/mL) and chloramphenicol (35  $\mu$ g/mL) overnight at 37°C with shaking. The overnight culture was transferred to 250 mL of sterile terrific broth medium (TB) (Sambrook et al., 1989) containing the antibiotics described above and incubated at 37°C with shaking until the OD<sub>600</sub> reached 0.5. A sample of non-induced bacteria was removed, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG; 0.5 mM) was added to the remaining culture and incubated for a further 4 h at 20°C with shaking. Non-induced and induced cells were harvested and stored at -80°C until further processing.

Soluble proteins were released from the induced bacteria by resuspending the cells in 12 mL of native lysis buffer [50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl and 10 mM imidazole (Sigma), pH 8.0] and disrupting cell walls by incubation with lysozyme (Sigma; 1 mg/mL) on ice for 30 min followed by sonication on ice. Cellular debris and insoluble protein were pelleted by centrifugation (10,000 $\times$  g) for 30 min at 4°C and the supernatant was stored at -80°C until further processing.

rIFN- $\gamma$ 2 was purified under native conditions on an immobilised metal affinity column (Bio-Scale Mini Profinity IMAC cartridge, Bio-Rad Laboratories, Gladesville, NSW, Australia) followed by desalting on a desalt column (Bio-Scale Mini Bio-Gel P-6 desalting cartridges, Bio-Rad). Protein purification was performed using the Profinity protein purification system following the manufacturer's instructions (Profinity protein purification system, Bio-Rad). To determine the exact mass of the expressed protein, 8  $\mu$ L of the purified protein was loaded onto a C18

column (Zorbax 80SB-C18, 74µm x 43mm, Agilent, Forest Hill, Victoria, Australia) and analysed using a linear ion trap mass spectrometer (LC/MSD Trap XCT Plus, Agilent). Full mass LC/MS data were manually analysed using DataAnalysis 6300 Series Ion Trap LC/MS Software 6.1 (version 3.4, Bruker Daltonik GmbH, Bremen, Germany).

The non-induced cell pellet, soluble bacterial lysate and affinity purified rIFN-γ2 were diluted with SDS-PAGE buffer [200 mM Tris (pH 6.8). 400 mM DTT. 8% SDS, 50% glycerol and 0.4% bromophenol blue (Sigma)], boiled for 5 min, electrophoresed through a 12% SDS-PAGE gel and stained with Coomassie brilliant blue R-250 (Sigma). In-gel digestion for protein identification was performed as previously described (Shevchenko et al., 2006). Briefly, the gel band containing purified rIFN-γ2 was excised, washed in 50% acetonitrile/NH<sub>4</sub>HCO<sub>3</sub>, reduced with 20mM DTT followed by alkylation in 100mM iodoacetamide (Sigma). In-gel digestion was performed using sequencing grade trypsin (Sigma) at a final concentration of 10 µg/mL in 25mM NH<sub>4</sub>HCO<sub>3</sub>. Peptides extracted after overnight digestion were separated on a C18 column (Zorbax 80SB-C18, 74µm x 43mm, Agilent) and analysed using a linear ion trap mass spectrometer (LC/MSD Trap XCT Plus, Agilent). MS/MS data were used to search the NCBI non-redundant protein database using Mascot software (version 2.2, [www.matrixscience.com](http://www.matrixscience.com), Matrix Science, Boston, USA).

Purified rIFN-γ2 was assessed for bacterial lipopolysaccharide (LPS) contamination using the E-TOXATE (*Limulus* amoebocyte lysate) test kit (Sigma) as per manufacturer's instructions.

### **7.3.3 *Ex vivo* stimulation of head kidney leucocytes with recombinant interferon- $\gamma$**

Atlantic salmon were euthanised (5 mL/25 L Aqui-S NZ, Lower Hutt, New Zealand) and head kidneys were removed aseptically, teased through a 100  $\mu$ m stainless steel mesh and placed in L-15 medium with L-glutamine (300 mg/L) (Gibco, Mount Waverley, Australia) supplemented with 2% foetal calf serum (Sigma), heparin (10 U/mL), penicillin (100 U/mL) and streptomycin (0.1 mg/mL). For *ex vivo* stimulation of head kidney leucocytes (HKL), cells were placed in culture at  $1 \times 10^6$  cells/mL and stimulated with or without rIFN- $\gamma$ 2 at 1, 10 or 100 ng/mL or phytohaemagglutinin-L (PHA-L, Sigma) at 10  $\mu$ g/mL for 4 h at 18°C. Subsequently, HKL were prepared as described above, stimulated with or without rIFN- $\gamma$ 2 (100 ng/mL) and maintained for 4, 6 and 8 h post-stimulation at 18°C.

### **7.3.4 Induction of amoebic gill disease and confirmation of the aetiological agent**

AGD-affected Atlantic salmon were obtained from a tank-based population of fish maintained at the School of Aquaculture, University of Tasmania. Unaffected fish were maintained under the same conditions. Fish were checked regularly and fish presumptively diagnosed with AGD were euthanized (5 mL/25 L Aqui-S NZ). The surfaces of the anterior and posterior hemibranchs were gently wiped using a sterile cotton-tip swab. The swab was then immersed in 500  $\mu$ L sterile phosphate buffered saline (PBS) and processed for PCR confirmation of *N. perurans* as the aetiological agent of AGD as previously described (Young et al., 2008b). The second left anterior hemibranch was removed aseptically, fixed, processed, sectioned (5  $\mu$ m) and stained

with haematoxylin and eosin following routine histological procedures. AGD gill lesions were identified by histological examination using established criteria (Dyková and Novoa, 2001). The severity of AGD was expressed as the proportion of gill filaments exhibiting AGD lesions within each section (Adams and Nowak, 2004b). The presence of *N. perurans* was then confirmed on three AGD-affected gill hemibranchs using *in situ* hybridisation with *N. perurans* and *Neoparamoeba pemaquidensis*-specific probes (Young et al., 2007).

### **7.3.5 *Ex vivo* stimulation of gill tissues with recombinant interferon- $\gamma$**

The gill baskets of Atlantic salmon were removed aseptically and placed directly into ice-cold PBS containing heparin (10 U/mL), penicillin (100 U/mL) and streptomycin (0.1 mg/mL) (PBS<sup>+</sup>). The gill basket was washed twice with fresh PBS<sup>+</sup> then individual gill hemibranchs were separated and washed again with fresh PBS<sup>+</sup>. The gill samples were then excised under a stereomicroscope to isolate either normal or AGD-affected tissues. AGD-affected tissues consisted of gill lesions from AGD-affected fish (AGD<sup>+</sup> L) whilst normal gill tissues were excised from similar regions of the gill in AGD-naive fish (AGD<sup>-</sup> NL) that did not exhibit any evidence of AGD pathology. Cells were dissociated from excised gill tissues by incubation with PBS<sup>+</sup> and dispase (Gibco, 2.4 U/mL) for 30 min at 37°C with gentle shaking. Gill tissues were finally teased gently through a 100  $\mu$ m stainless steel mesh and placed in the supplemented L-15 medium described above. For *ex vivo* stimulation of AGD<sup>-</sup> NL tissue, cells were placed in culture at  $1 \times 10^6$  cells/mL and initially stimulated with or without rIFN- $\gamma$ 2 (100 ng/mL) for 2, 4, 6 and 8 h at 18°C. Subsequently, AGD<sup>+</sup> L and AGD<sup>-</sup> NL cells were prepared as described above, stimulated with or without rIFN- $\gamma$ 2 (100 ng/mL) and cultured for 6 h at 18°C.

### **7.3.6 Identification and comparison of Atlantic salmon transcripts**

Since the complete gene sequences for interferon regulatory factor-2 (IRF-2) and interferon regulatory factor-8 (IRF-8) were unavailable, full length, Phrap-assembled, contiguous Atlantic salmon sequences were obtained by querying the ongoing Genomic Research on Atlantic salmon Project (GRASP) consortium clustering database (Rise et al., 2004b) using rainbow trout, *Oncorhynchus mykiss*, or Atlantic salmon expressed sequence tags (ESTs). Comparative alignment of the predicted amino acid translations were performed using MAFFT L-INS-i sequence alignment by means of local pairwise alignment information (Katoh et al., 2005). Amino acid translations were submitted to the PredictProtein server (Rost et al., 2004) which predicted conserved motifs and secondary structure. Identity and similarity matrices were determined in BioEdit (Hall, 1999) using a BLOSUM62 amino acid substitution matrix.

### **7.3.7 RNA extraction and cDNA synthesis for quantitative reverse-transcription PCR (qRT-PCR) assays**

Total RNA was purified from each sample and DNase I treated as described above. Total RNA was reverse transcribed from HKLs (800 ng) and gill tissues (400 ng) as described above with the exception that only the oligo(dT)<sub>20</sub> (Invitrogen) oligonucleotide was used. Three undiluted samples of cDNA from AGD<sup>+</sup> L and AGD<sup>-</sup> NL or rIFN- $\gamma$ 2-stimulated HKL were pooled in equal proportions and used as cDNA template to calculate the amplification efficiency of each gene of interest.

The relative expression of mRNA from IFN- $\gamma$ -inducible genes following stimulation with rIFN- $\gamma$  was assessed by qRT-PCR. PCR oligonucleotides were designed against Atlantic salmon transcripts of interest and to anneal at 55°C (Table 7.1). In some cases, oligonucleotides were available for genes including RNA polymerase II (RPL2) (Jørgensen et al., 2006a),  $\beta$ -2-microglobulin ( $\beta$ 2m) (Young et al., 2008a), MHC class I (Jørgensen et al., 2006a), interferon regulatory factor 1 (IRF-1) (Young et al., 2008a) and elongation factor 1- $\alpha$  (EF1 $\alpha$ ) (Young et al., 2008a). In the case of the MHC class I gene, the oligonucleotides amplified a portion of the conserved  $\alpha$ 3-region of the *Sasa*-UBA locus.

PCR amplification was performed using a real-time PCR detection system (IQ5, Bio-Rad) as previously described (Young et al., 2008a). The mean  $C_T$  deviation between the treatment and control groups was determined and normalised against two reference genes (Table 7.1). The relative stability of reference genes was calculated as a gene expression stability measure ( $M$ ) using GeNorm software (Vandesompele et al., 2002). The relative expression (fold-change) of each gene of interest was determined after empirically-derived PCR efficiencies were used to correct the data using Relative Expression Software Tool software (REST-384 version 2) (Pfaffl et al., 2002). The relative expression of the genes of interest was subsequently tested for significance ( $p = 0.05$ ) by a pair-wise fixed reallocation randomisation test (Pfaffl et al., 2002).

## 7.4 Results

### 7.4.1 Molecular cloning of a second isoform of Atlantic salmon interferon- $\gamma$

A full-length contiguous sequence was obtained with high homology to IFN- $\gamma$  transcripts from rainbow trout, *O. mykiss* (*Onmy*-IFN- $\gamma$ , GenBank accession number: AJ616215) and Atlantic salmon (*Sasa*-IFN- $\gamma$ 1, GenBank: AJ841811).

Oligonucleotides based on this initial sequence were designed for RACE. A contiguous sequence amplified from Atlantic salmon gill mRNA was obtained and the full-length transcript was designated IFN- $\gamma$  isoform 2 (*Sasa*-IFN- $\gamma$ 2, GenBank: FJ263446). The complete transcript is 1175 bases with an open reading frame (ORF) of 540 bases showing 96.5% identity to *Onmy*-IFN- $\gamma$  and 96.3% identity to *Sasa*-IFN- $\gamma$ 1 (Fig. 7.1A). The 156 base 5' untranslated region (UTR) is homologous to *Sasa*-IFN- $\gamma$ 1 (95.2% identity) while the 479 base 3' UTR was variable in nucleotide composition between the two Atlantic salmon IFN- $\gamma$  isoforms (80.8% identity to *Sasa*-IFN- $\gamma$ 1) but both possess 6 mRNA (ATTTA) instability motifs, a polyadenylation signal sequence (AATAAA) 16 bases upstream of the polyA tail (Fig. 7.1A) and a similar A/T content (*Sasa*-IFN- $\gamma$ 1: 66.3% and *Sasa*-IFN- $\gamma$ 2: 66.7%).

Nucleotide variations within the ORF resulted in 11 amino acid substitutions in the 180 predicted amino acid translations between *Sasa*-IFN- $\gamma$ 1 and *Sasa*-IFN- $\gamma$ 2 (Fig. 7.1B). These substitutions did not affect the predicted signal peptide, N-glycosylation sites or putative alpha helices (Fig. 7.1B). Similarly, the putative IFN- $\gamma$  family signature [I/V]-Q-X-[K/Q]-A-X<sub>2</sub>-E-[L/F]-X<sub>2</sub>-[I/V] (Zou et al., 2005) and the predicted nuclear localisation signal (NLS), an arginine (R) and lysine (K)-rich C-terminal domain are retained in all salmonid IFN- $\gamma$  amino acid translations (Fig.

7.1B). The predicted amino acid residues of Sasa-IFN- $\gamma$ 2 are most similar to *Onmy*-IFN- $\gamma$  and *Sasa*-IFN- $\gamma$ 1 (Fig. 7.1B) and share between 26.7-33.2 % identity and between 42.1-51.6% similarity to known teleost IFN- $\gamma$  amino acid translations (Fig. 7.1B). A predicted cleavage site between alanine<sup>24</sup> and alanine<sup>25</sup> would release a *Sasa*-IFN- $\gamma$ 2 mature peptide with a predicted molecular weight of 18.45 kDaltons (kDa).

Figure 7.1. Identification of a second isoform of Atlantic salmon (*Salmo salar*) interferon- $\gamma$  (IFN- $\gamma$ 2). A. Nucleotide and predicted amino acid sequence Atlantic salmon IFN- $\gamma$  isoform 2 mRNA (GenBank accession number: FJ263446). The amino acid translations are shown below the coding region and the signal peptide (dark-grey shaded text), putative IFN- $\gamma$  family signature [I/V]-Q-X-[K/Q]-A-X<sub>2</sub>-E-[L/F]-X<sub>2</sub>-[I/V] (light-grey shaded text), arginine (R)/lysine (K)-rich nuclear localisation signal (NLS) (bold italic), polyadenylation signal (bold and underlined) and ATTTA elements associated with mRNA instability (bold) are highlighted. The amino acid sequences displayed in Fig. 7.2B with peptide matches ( $p < 0.05$ ) obtained using Mascot on the LC/MS/MS analysed rIFN- $\gamma$  in-gel digest are underlined. B. Comparison of predicted IFN- $\gamma$ 2 amino acid translations from Atlantic salmon (bold title), human and various teleost fish. Dashes denote gaps. Amino acid conservation is denoted by an asterisk (\*), whereas colons (:) and full stops (.) represent a high and low degree of similarity respectively. A comparison of percentage identity (%ID) and similarity (%SIM) of IFN- $\gamma$ 2 amino acid translation to other aligned sequences is displayed. The predicted signal peptide (dark-grey shaded text), the putative IFN- $\gamma$  family signature (boxed) and NLS (bold and italic) are highlighted. For the salmonid IFN- $\gamma$  amino acid translations, predicted N-glycosylation (N-Gly) (underlined) and alpha helices (light-grey shaded text) are highlighted.





## **7.4.2 Expression and activity of rIFN- $\gamma$**

### **7.4.2.1 Purity and confirmation of recombinant *Sasa*-IFN- $\gamma$ 2 identity**

No differences in biological activity were predicted between the two isoforms of *Sasa*-IFN- $\gamma$ , therefore the transcript that was successfully amplified by RACE from Atlantic salmon gill tissues (*Sasa*-IFN- $\gamma$ 2) and shared highest homology to the biologically active *Onmy*-IFN- $\gamma$  (Zou et al., 2005) was selected for subsequent functional studies. The putative mature peptide was expressed as an N-terminal 6 $\times$  His-tagged fusion protein in *E. coli* Rosetta cells and purified under native conditions by metal affinity chromatography. This produced a 21.12 kDa protein, as estimated by MS (data not shown), comprising of the predicted mature peptide (18.45 kDa) and the pQE30 UA vector-encoded tag (approximately 2.7 kDa) (Fig. 7.2A). The rIFN- $\gamma$ 2 was confirmed by both, nucleotide sequencing (data not shown) and LC/MS/MS analysis of the in-gel digested SDS-PAGE gel band. Four tryptic peptides homologous ( $p < 0.05$ ) to *Sasa*-IFN- $\gamma$ 1 were recovered confirming the identity of the expressed protein (Fig. 7.2B). The purified rIFN- $\gamma$ 2 contained  $< 0.1$  ng LPS per 100  $\mu$ g rIFN- $\gamma$ 2 according to the *Limulus* haemocyte agglutination assay, an LPS concentration below the minimum concentration that stimulates an immunological response in salmonid fish (Goetz et al., 2004; Hong et al., 2001; Zou et al., 2005; Zou et al., 2003).

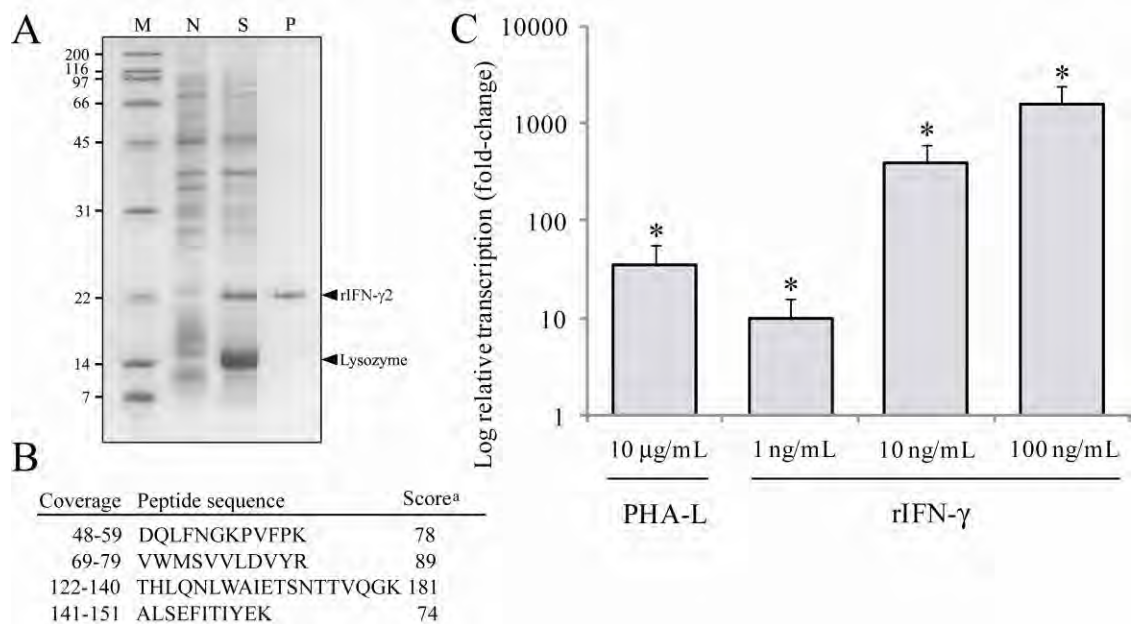


Figure 7.2. Expression, purification and activity of Atlantic salmon (*Salmo salar*) recombinant interferon- $\gamma$  isoform 2 (rIFN- $\gamma$ 2). A. Purity of Atlantic salmon rIFN- $\gamma$ 2 assessed by SDS-PAGE. The 6 $\times$  histidine-tagged rIFN- $\gamma$  mature peptide was ligated into a pQE30 UA expression vector and expression was induced in *E. coli* (Rosetta strain) using isopropyl  $\beta$ -D-thiogalactoside (IPTG). rIFN- $\gamma$ 2 was affinity purified (P) from the soluble bacterial lysate proteins (S) on a metal affinity column using the Profinia protein purification system. Protein samples were reduced in the presence of DTT, electrophoresed through a 12% gradient polyacrylamide gel and Coomassie blue stained. Lysates from bacteria that were not induced to express rIFN- $\gamma$ 2 (N). Molecular weight marker (M). B. The identity of rIFN- $\gamma$ 2 was confirmed by sequencing independent regions of the protein using liquid chromatography/mass spectrometry (LC/MS/MS) on the trypsin digested SDS-PAGE gel band. LC/MS/MS data were searched against the NCBI protein database ( $p < 0.05$ ). Four tryptic peptides homologous to Atlantic salmon IFN- $\gamma$  (GenBank protein accession number CAH56503) were obtained. The probability based mowse scores for the individual peptides are shown (<sup>a</sup>) Individual ions scores  $> 61$  indicate identity or extensive homology ( $p < 0.05$ ). The position of amino acid residues are displayed on the predicted open reading frame in Figure 7.1A. C. Stimulation of head kidney leucocytes (HKL) with 3 different concentrations of rIFN- $\gamma$  successfully induced the gene expression of IFN- $\gamma$ -inducible CXCL10-like protein ( $\gamma$ IP-10). Relative transcription (fold-change) of  $\gamma$ IP-10 in HKL stimulated *ex vivo* for 4 hours with 3 concentrations of rIFN- $\gamma$  and 10  $\mu$ g/mL phytohaemagglutinin-L (PHA-L) by quantitative real-time PCR (qRT-PCR) analysis of mRNA. Data are presented as mean fold-change ( $\pm$ standard error) in stimulated HKL ( $n = 3$  wells) compared to non-stimulated HKL ( $n = 3$  wells).  $\gamma$ IP-10 qRT-PCR threshold values were normalised against elongation factor 1 $\alpha$  and RNA polymerase II. Significant differences ( $p < 0.05$ ) in gene regulation are indicated with an asterisk (\*).

#### **7.4.2.2 Transcriptional response of primary head kidney cells to *ex vivo* stimulation with rIFN- $\gamma$**

The relative expression of mRNA from HKLs was assessed by qRT-PCR using two reference genes, EF1 $\alpha$  (0.63 M score) and RPL2 (0.57 M score). In salmonids, recombinant rainbow trout IFN- $\gamma$  is a potent activator of the chemokine, IFN- $\gamma$ -inducible CXCL10-like protein ( $\gamma$ IP-10) (Martin et al., 2007c; Zou et al., 2005). Therefore,  $\gamma$ IP-10 was used as an indicator of rIFN- $\gamma$ 2-mediated agonism. The deduced amino acid residues for Atlantic salmon  $\gamma$ IP-10 retain 4 cysteine residues including a CXC repeat at the N-terminus of the mature peptide and shared 85% identity and 89% similarity to the rainbow trout  $\gamma$ IP-10 (Laing et al., 2002). Primary head kidney leucocytes stimulated with either PHA-L, which served as a positive control, or rIFN- $\gamma$ 2 at 1, 10 or 100 ng/mL produced a dose-dependent response in the expression of  $\gamma$ IP-10 relative to a non-induced control at 4 hours post-stimulation (Fig. 7.2C). rIFN- $\gamma$  at 100 ng/mL induced the greatest fold-change in the expression of  $\gamma$ IP-10 ( $1574.1 \pm 866.1$ , mean  $\pm$  standard error) and was therefore used in the subsequent time-course study. IRF-1 is activated by IFN- $\gamma$  via the Janus kinase (JAK) JAK/signal transducers and activators of transcription (STAT) signal pathway (STAT1) and activates the expression of MHC class I and other IFN- $\gamma$  inducible genes when bound to interferon-stimulated response elements (ISRE) within their promoter region (Hobart et al., 1997). MHC I can also be directly induced by IFN- $\gamma$  via the JAK/STAT pathway through STAT dimerisation and translocation to the nucleus and binding to gamma-interferon activate sequence (GAS) response elements in the promoter of MHC class I (Dijkstra et al., 2003b; Schroder et al., 2004). A significant response in  $\gamma$ IP-10, IRF-1 and MHC I

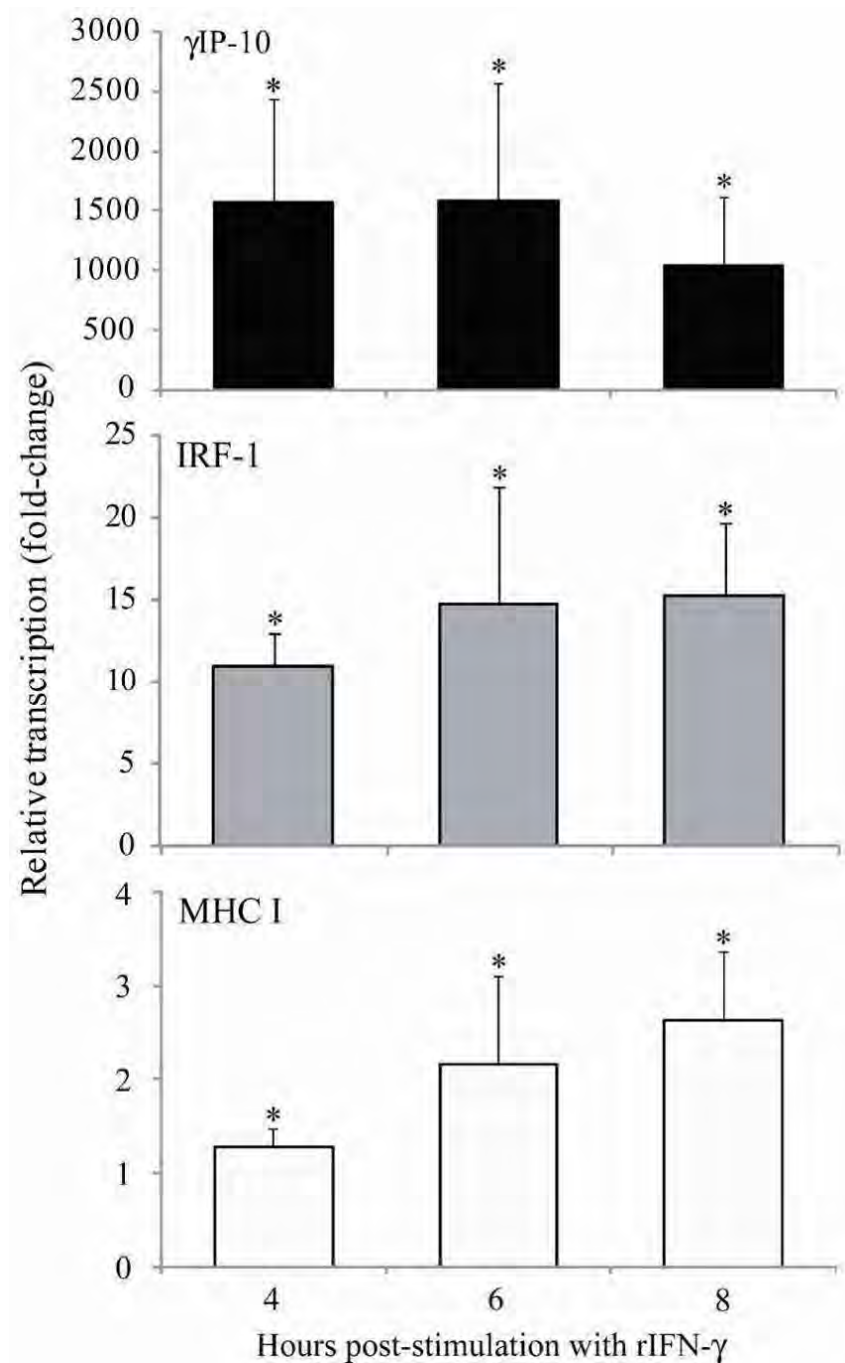


Figure 7.3. Recombinant Atlantic salmon interferon- $\gamma$  isoform 2 (rIFN- $\gamma$ 2) induces the expression of MHC class I-associated genes in Atlantic salmon (*Salmo salar*) head kidney leucocytes (HKL) within eight hours post-stimulation. Time course of the relative transcription (fold-change) in IFN- $\gamma$  inducible genes in Atlantic salmon HKL stimulated with rIFN- $\gamma$ 2. Data are presented as mean fold-change ( $\pm$ standard error) in HKL stimulated with 100 ng/ml rIFN- $\gamma$  (n = 3 wells) compared to non-stimulated HKL (n = 3 wells). Significant differences (p < 0.05) in gene regulation are indicated with an asterisk (\*). Genes examined were IFN- $\gamma$ -inducible CXCL10-like protein ( $\gamma$ IP-10), interferon regulatory factor I (IRF-1) and MHC I subunit  $\alpha$ 3 of the *Sasa*-UBA locus (MHC I). qRT-PCR threshold values for each gene were normalised against elongation factor 1 $\alpha$  and RNA polymerase II.

relative to non-induced control cells was observed at 4, 6 and 8 hours post-stimulation with rIFN- $\gamma$ 2 (Fig. 7.3). While the fold-change in  $\gamma$ IP-10 response was greatest at 4 ( $1574.1 \pm 866.1$ ) and 6 ( $1587.9 \pm 973.0$ ) hours post-stimulation (Fig. 7.4) the response of IRF-1 ( $14.8 \pm 7.0$ ,  $15.3 \pm 4.3$ ) and MHC I ( $2.2 \pm 0.9$ ,  $2.6 \pm 0.7$ ) was greatest after 6 and 8 h respectively.

#### **7.4.2.3 Viability of gills cells during *ex vivo* culture and their $\gamma$ IP-10 response following stimulation with rIFN- $\gamma$**

No changes in primary gill cell morphology or loss of cell viability (assessed by 0.2% Trypan blue exclusion) was apparent after 8 h incubation at 18°C (data not shown). While cells remained morphologically intact in cultures maintained for 12 h and 24 h, cell viability appeared to be compromised (data not shown). The relative expression of mRNA from Atlantic salmon gill cells was assessed by qRT-PCR using two reference genes, EF1 $\alpha$  (0.52 *M* score) and RPL2 (0.48 *M* score). A significant  $\gamma$ IP-10 response was observed in Atlantic salmon gill cells stimulated with rIFN- $\gamma$ 2 after 4 h ( $2.7 \pm 1.1$ ) and 6 h ( $4.3 \pm 2.9$ ) (Fig. 7.4). Following 8 h post-stimulation with rIFN- $\gamma$  the gill cells appeared normal with no loss of cell viability (data not shown) yet they responded inconsistently to IFN- $\gamma$  treatment as indicated by the high variability in  $\gamma$ IP-10 expression within the treatment replicates (Fig. 7.4). Therefore, to limit variability between treatment replicates subsequent trials using primary gill cell cultures were restricted to 6 h post-stimulation with rIFN- $\gamma$ 2.



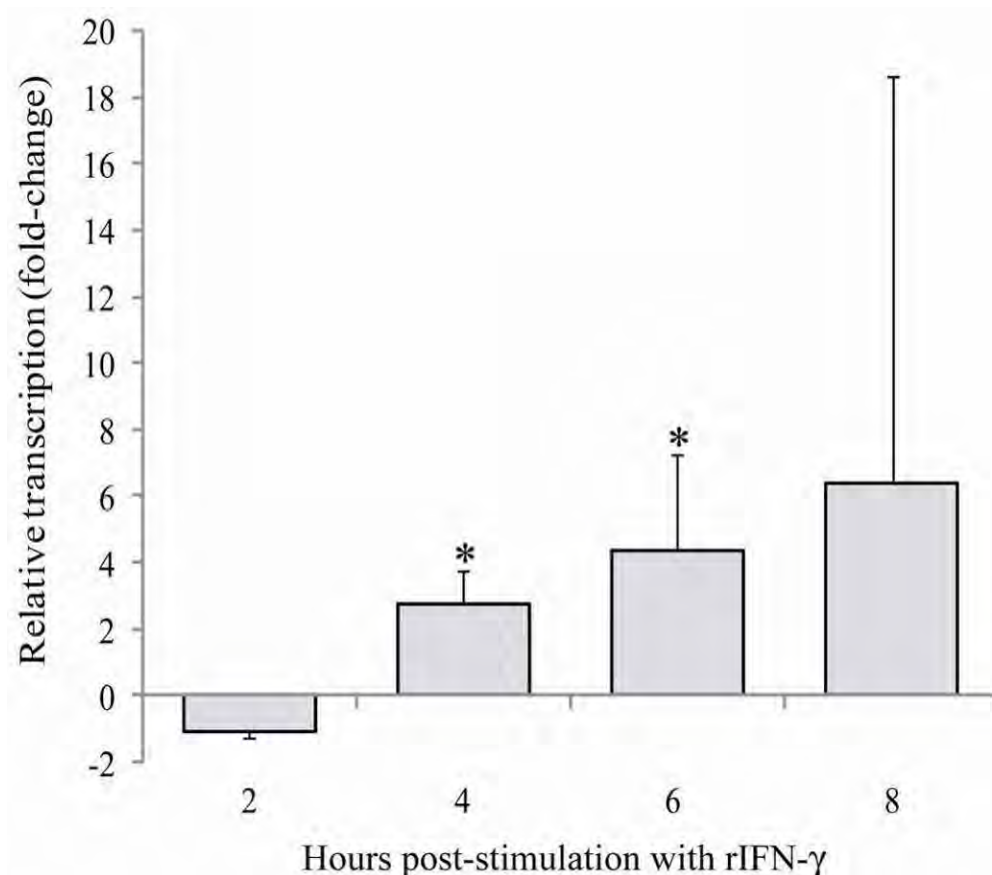


Figure 7.4. Recombinant Atlantic salmon (*Salmo salar*) interferon- $\gamma$  isoform 2 (rIFN- $\gamma$ 2) induced the most stable, significant expression of IFN- $\gamma$ -inducible CXCL10-like protein ( $\gamma$ IP-10) in Atlantic salmon gill cells after six hours post-stimulation. Time course of the relative transcription (fold-change) of  $\gamma$ IP-10 in Atlantic salmon gill cells stimulated *ex vivo* with rIFN- $\gamma$ 2 analysed by quantitative real-time PCR (qRT-PCR) analysis of mRNA. Data are presented as mean fold-change ( $\pm$ standard error) in stimulated gill cells ( $n = 3$  wells) compared to non-stimulated gill cells ( $n = 3$  wells).  $\gamma$ IP-10 qRT-PCR threshold values were normalised against elongation factor 1 $\alpha$  and RNA polymerase II. Significant differences ( $p < 0.05$ ) in gene regulation are indicated with an asterisk (\*).

### 7.4.3 Induction of AGD in Atlantic salmon and identification of the aetiological agent

Numerous lesions were visible across the gills of AGD-affected Atlantic salmon consistent with other cases of AGD (Dyková et al., 1995; Kent et al., 1988; Munday et al., 2001; Rodger and McArdle, 1996; Roubal et al., 1989). The most prominent feature was the extensive hyperplasia of epithelial-like cells, resulting in the fusion of secondary lamellae (Fig. 7.5A). The severity of AGD in the gills of Atlantic salmon

used for *ex vivo* IFN- $\gamma$  treatment ( $n = 7$  fish) was high with 74 to 97% of gill filaments affected by AGD in each histological section examined. In gill tissues examined from three fish only the *N. perurans*-specific ISH probe hybridised with the amoebae associated with AGD-lesions (Fig. 7.5A and 7.5A inset). *N. pemaquidensis* has not been directly associated with AGD and on serially-sectioned gill tissues from the same fish the *N. pemaquidensis* probe did not hybridise with amoebae present in gill tissues and constituted a negative probe control (Fig. 7.5A inset). *N. perurans*-specific oligonucleotides successfully PCR-amplified genomic DNA extracted from all AGD-affected fish gill swabs (Fig. 7.5B). No PCR amplicons were produced from genomic DNA extracted from AGD-naive gill swabs (data not shown). Gross and histopathological examination of the gills of AGD-naive fish revealed a normal gill structure with no visible signs of hyperplastic tissue (data not shown).

#### **7.4.4 MHC class I gene response of AGD-affected primary gill cells to stimulation with rIFN- $\gamma$ *ex vivo***

The relative expression of mRNA from AGD-affected Atlantic salmon gill cells was assessed by qRT-PCR using two reference genes, EF1 $\alpha$  (0.51 M score) and RPL2 (0.51 M score). In the first instance, AGD-affected gill lesion cells (AGD<sup>+</sup> L) were compared to AGD-naive non lesion gill cells (AGD<sup>-</sup> NL) to confirm that: 1) the MHC I antigen presentation pathway was down-regulated as described in Chapter 6, and 2) the MHC I antigen presentation pathway was still down-regulated after 6 h *ex vivo* culture (Fig. 7.6A). A coordinated down-regulation of MHC I ( $-5.5 \pm 1.8$ ) and  $\beta 2m$  ( $-4.7 \pm 1.7$ ) was observed in AGD<sup>+</sup> L cells as previously described (Young et al., 2008a). Associated with this was the co-ordinated down-



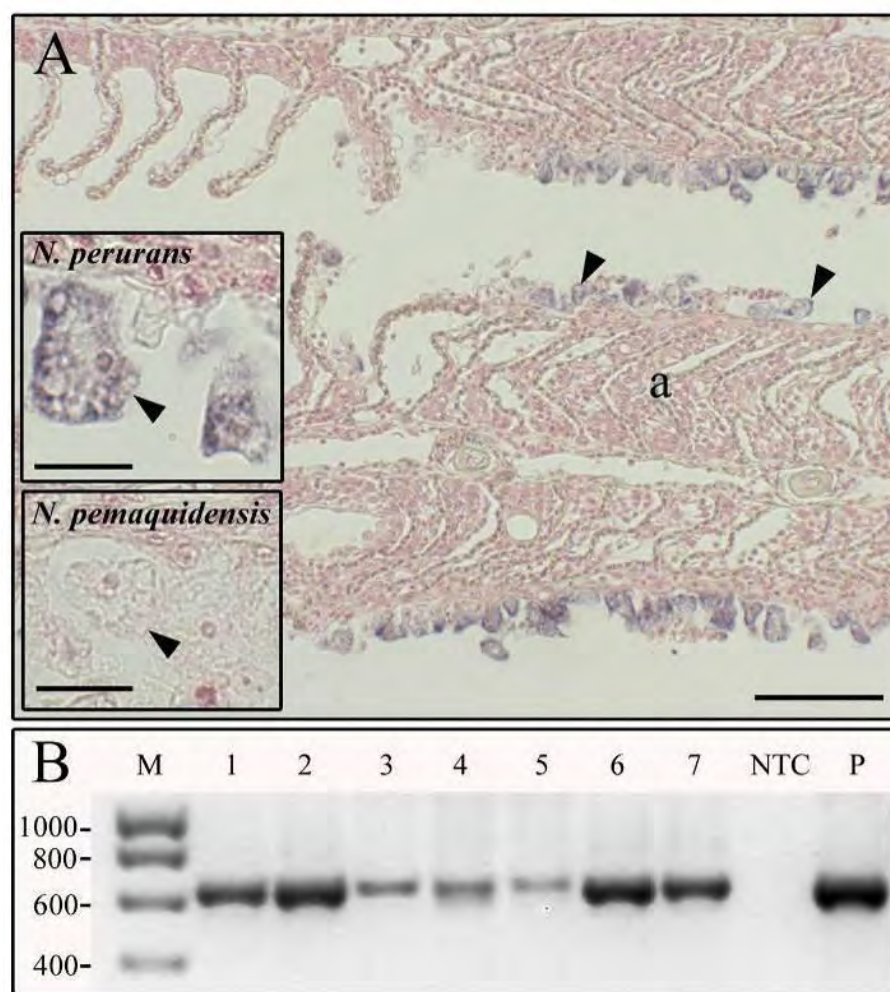
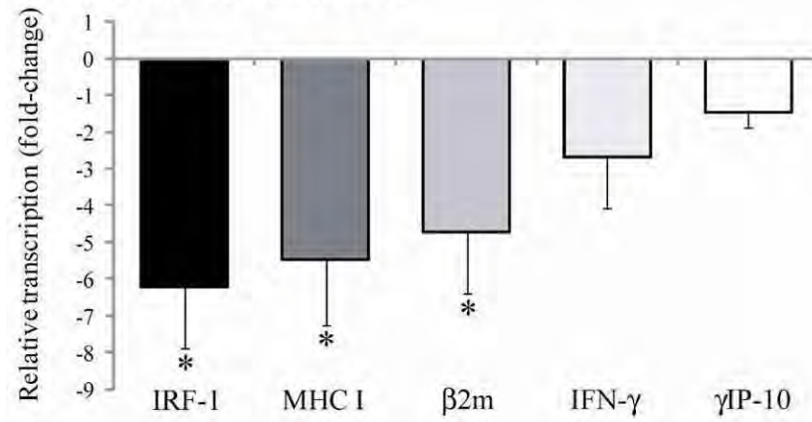
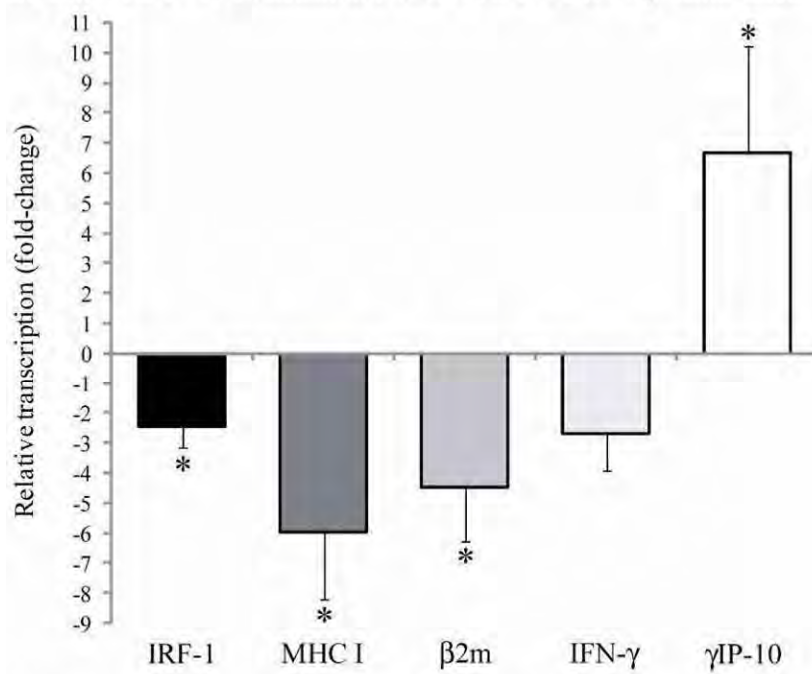


Figure 7.5. Confirmation that Atlantic salmon (*Salmo salar*) were affected by *Neoparamoeba perurans*-induced amoebic gill disease (AGD). A. Confirmation that AGD was associated with the attachment of *N. perurans* to affected gill tissues (a). Amoebae (arrows) identity was assessed by *in situ* hybridisation using an *N. perurans*-specific oligonucleotide probe. Bar = 100  $\mu$ m. Insert shows magnified amoebae assessed using the *N. perurans*-specific oligonucleotide probe or a *N. pemaquidensis*-specific oligonucleotide probe as a negative control. Bars = 20  $\mu$ m. B. Confirmation that *N. perurans* was associated with AGD-gill lesions in all affected Atlantic salmon used in this study. PCR amplification of a 636 bp region of the 18S rRNA gene using *N. perurans* oligonucleotides and genomic DNA taken from the gills of fish used in this study (lanes 1-7) that were presumptively diagnosed with AGD. Control PCR reactions included no DNA template control (NTC) and a positive control (P) consisting of purified plasmid DNA containing the entire 18S rRNA gene of *N. perurans* (GenBank accession number: EF216901).

A. Gill: AGD<sup>-</sup> NL vs. AGD<sup>+</sup> L



B. Gill: AGD<sup>-</sup> NL unstimulated vs. AGD<sup>+</sup> L IFN-γ stimulated



C. Gill, AGD<sup>+</sup> L: Unstimulated vs. IFN-γ stimulated

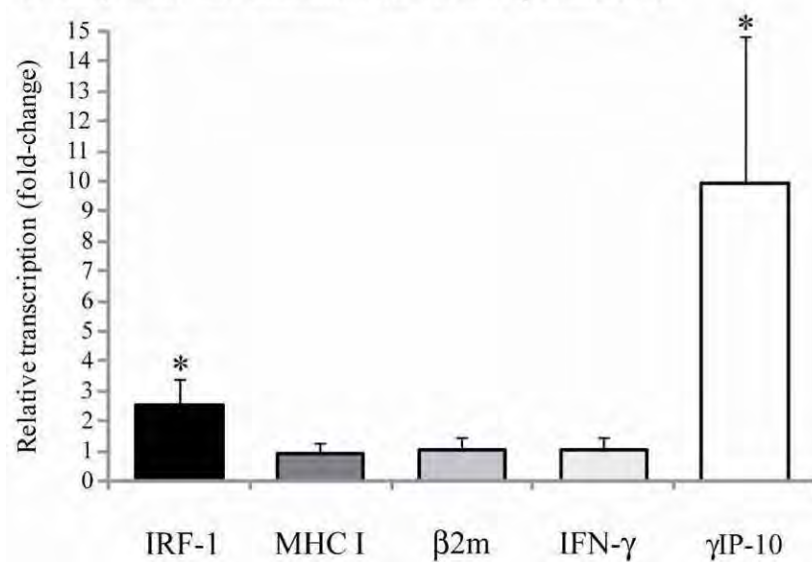


Figure 7.6. The expression of MHC class I (MHC I)-associated genes in amoebic gill disease (AGD)-affected Atlantic salmon (*Salmo salar*) gill cells is not restored by stimulation with recombinant interferon- $\gamma$  isoform 2 (rIFN- $\gamma$ 2). Transcriptional response of IFN- $\gamma$  and IFN- $\gamma$ -inducible genes associated with the MHC I antigen processing and presentation pathway in AGD-affected Atlantic salmon gill cells stimulated for six hours with rIFN- $\gamma$ 2. The relative transcription (fold-change) of genes in the gill tissues were determined by quantitative real-time PCR (qRT-PCR) analysis of mRNA. Data are presented as mean fold-change ( $\pm$ standard error). qRT-PCR threshold values were normalised against elongation factor 1 $\alpha$  and RNA polymerase II. Significant differences ( $p < 0.05$ ) in gene regulation are indicated with an asterisk (\*). Genes examined were interferon regulatory factor I (IRF-1), MHC I subunit  $\alpha$ 3 of the *Sasa*-UBA locus (MHC I),  $\beta$ 2-microglobulin ( $\beta$ 2m) and IFN- $\gamma$ -inducible CXCL10-like protein ( $\gamma$ IP-10). A. Confirmation that MHC I pathway genes were down-regulated in the gills of amoebic gill disease (AGD)-affected fish by comparing transcriptional response in AGD lesion (AGD<sup>+</sup> L) tissue (n = seven fish) to AGD<sup>-</sup> non-lesion (NL) tissue (n = 7 fish). B. AGD<sup>+</sup> L (n = 7 fish) stimulated *ex vivo* with rIFN- $\gamma$ 2 (100 ng/mL) compared to unstimulated AGD<sup>-</sup> NL tissue (n = 7 fish). C. AGD<sup>+</sup> L (n = 7 fish) stimulated *ex vivo* with rIFN- $\gamma$ 2 (100 ng/mL) compared to unstimulated AGD<sup>+</sup> L tissue (n = 7 fish).

regulation in MHC I-associated transcription factor, IRF-1 ( $-6.2 \pm 1.7$ ), a variable IFN- $\gamma$  transcriptional response (high standard error) and stable  $\gamma$ IP-10 transcriptional response (approximately 1-fold change in gene expression and low standard error).

Following

6 h stimulation with rIFN- $\gamma$ 2 the genes associated with MHCI antigen presentation were still down-regulated (Fig. 7.6B). AGD<sup>+</sup> L gill cells were successfully stimulated specifically with rIFN- $\gamma$  as demonstrated by the transcriptional response of  $\gamma$ IP-10 in comparison to non-stimulated AGD<sup>-</sup> NL (Fig. 7.6B,  $6.7 \pm 3.5$ ) and AGD<sup>+</sup> L (Fig. 7.6C,  $9.9 \pm 4.9$ ) cells. Despite an up-regulation in IRF-1 ( $-2.4 \pm 0.7$ ) in AGD<sup>+</sup> L cells following stimulation with rIFN- $\gamma$ , there was no significant change in the expression of MHC I ( $-6.0 \pm 2.2$ ) and  $\beta$ 2m ( $-4.5 \pm 1.8$ ). The induction of IRF-1 can be observed as an up-regulation in AGD<sup>+</sup> L gills cells in response to rIFN- $\gamma$ 2 ( $2.6 \pm 0.9$ ) when compared to AGD<sup>+</sup> L non-stimulated cells (Fig. 7.6C).

#### **7.4.5 Identification of Atlantic salmon IRF-2 and IRF-8 transcripts and relative transcriptome response of IRF-1, IRF-2 and IRF-8 to simulation with rIFN $\gamma$**

Whilst IRF-1 is a transcription activator, IRF-2 and IRF-8 serve to repress IRF-1-induced transcription (Barber et al., 1995; Nelson et al., 1993). Therefore, IRF-2 and IRF-8 were examined in this study to determine whether the differential transcriptional response of these repressor genes influenced the transcriptional response of MHC I-associated genes. The deduced amino acid residues of the putative Atlantic salmon IRF-8 (*Sasa*-IRF-8) retain the penta-tryptophan repeat characteristic of members of the IRF family [Fig. 7.7A; (Jungwirth et al., 1995)]. *Sasa*-IRF-8 is highly conserved across the N-terminal penta-tryptophan repeat region when compared to the predicted amino acid translations of IRF-8 from Zebrafish (*Danio rerio*, 88.2% identity, 92.4% similarity), chicken (*Gallus gallus*, 87.4%, 93.3%) and human (*Homo sapiens*, 85.7 %, 92.4%) (Fig. 7.7A). Variability in the amino acid residues of *Sasa*-IRF-8 was more apparent across the C-terminal region but was still conserved with 39.0 - 58.6% identity and 59.2 – 72.4% similarity to the IRF-8 amino acid translations described above (Fig. 7.7A). Atlantic salmon IRF-2 (*Sasa*-IRF-2) was identified through homology searches using rainbow trout IRF-2 (*Onmy*-IRF-2) (Collet et al., 2003). The predicted amino acid translation of *Sasa*-IRF-2 retains the IRF family signature at the N-terminus similar to IRF-1: NH<sub>2</sub>-X<sub>10</sub>-W-X<sub>14</sub>-W-X<sub>11</sub>-W-X<sub>7</sub>-W-X<sub>11</sub>-W-X<sub>18</sub>-W (Collet et al., 2003; Harada et al., 1989). Finally, a comparison of the global identity and similarity of the predicted amino acid translations for *Sasa*-IRF-1, *Sasa*-IRF-2 and *Sasa*-IRF-8 demonstrates that they are indeed, three distinct members of the IRF family (Fig. 7.7B).

Initially, the response of IRF-2 and IRF-8 to stimulation with rIFN- $\gamma$ 2 was assessed in HKL (Fig. 7.8A). An IRF-2 ( $3.4 \pm 1.5$ ) and IRF-8 ( $4.3 \pm 1.8$ ) transcriptional response to rIFN- $\gamma$ 2 was observed in HKLs, approximately 3 to 4 fold-less than the IRF-1 transcriptional response. Subsequently, AGD<sup>+</sup> L gill cells were compared to AGD<sup>-</sup> NL gill cells to determine the IRF-2 and IRF-8 transcriptional response in AGD-affected gill cells after 6 h *ex vivo* culture (Fig. 7.8B). IRF-8 was not differentially regulated in AGD<sup>+</sup> L cells in relation to AGD<sup>-</sup> NL cells, whilst IRF-2 was down-regulated ( $-4.6 \pm 1.2$ ) in contrast to previous findings (Young et al., 2008a). When rIFN- $\gamma$ 2-stimulated AGD<sup>+</sup> L cells were compared to non-stimulated AGD<sup>-</sup> NL cells IRF-2 remained down-regulated (Fig. 7.8C;  $-5.8 \pm 1.7$ ) shifting the ratio of IRF-1/IRF-2 from 1.4 to 2.4 post-stimulation with rIFN- $\gamma$  and there was no change in the IRF-8 transcriptional response. The IRF-2 and IRF-8 transcriptional response was explored further by comparing AGD<sup>+</sup>L stimulated cells with AGD<sup>+</sup>L non-stimulated cells (Fig. 7.8D). rIFN- $\gamma$ 2 stimulated an up-regulation in the IRF-1 ( $2.6 \pm 0.9$ ) and IRF-8 ( $1.8 \pm 0.6$ ) in AGD<sup>+</sup>L gill cells.



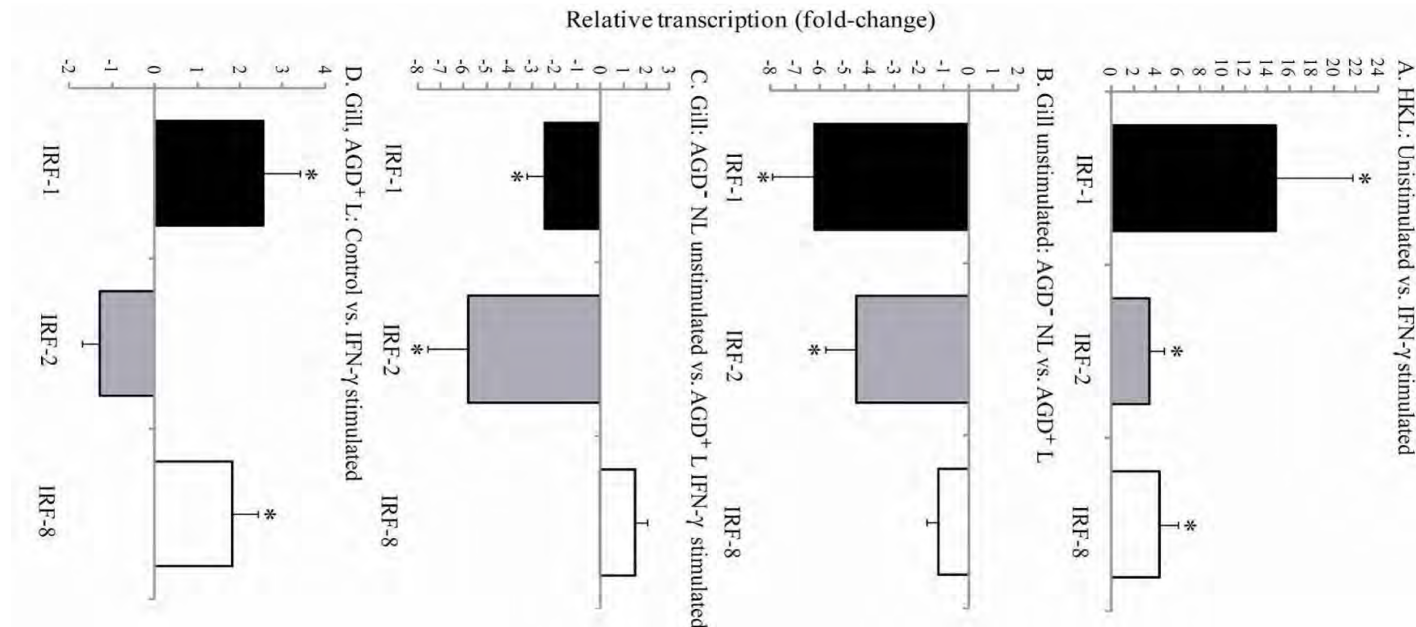


Figure 7.8. The ratio of interferon regulatory factor 1, 2 and 8 is altered in amoebic gill disease (AGD)-affected Atlantic salmon (*Salmon salar*) gill cells following stimulation with recombinant Atlantic salmon interferon- $\gamma$  isoform 2 (rIFN- $\gamma$ 2). Transcriptional response of interferon regulatory factor genes in AGD-affected Atlantic salmon gill cells stimulated for six hours with rIFN- $\gamma$ 2. Relative transcription (fold change) of genes in the gill cells was determined by quantitative real-time PCR (qRT-PCR) analysis of mRNA. Data are presented as mean fold-change ( $\pm$ standard error) in each gene. qRT-PCR threshold values were normalised against elongation factor 1 $\alpha$  and RNA polymerase II. Significant differences ( $p < 0.05$ ) in gene regulation are indicated with an asterisk (\*). Genes examined were interferon regulatory factor-1 (IRF-1), interferon regulatory factor-2 (IRF-2) and interferon regulatory factor-8 (IRF-8). A. Head kidney leucocytes (HKL) stimulated with 100 ng/mL rIFN- $\gamma$ 2 compared to unstimulated HKL ( $n = 3$  wells). B. AGD-affected lesion tissue (AGD<sup>+</sup> L) compared to AGD<sup>-</sup> non-lesion (NL) tissue ( $n = 7$  fish). C. AGD<sup>+</sup> L ( $n = 7$  fish) stimulated *ex vivo* with rIFN- $\gamma$ 2 (100 ng/mL) compared to unstimulated AGD<sup>-</sup> NL ( $n = 7$  fish). D. AGD<sup>+</sup> L ( $n = 7$  fish) stimulated *ex vivo* with rIFN- $\gamma$ 2 (100 ng/mL) compared to unstimulated AGD<sup>+</sup> L ( $n = 7$  fish).



#### 7.4.6 MHC class I gene response of primary gill cells from healthy Atlantic salmon to stimulation with rIFN- $\gamma$ *ex vivo*

It was unclear why the transcriptional response of MHC I antigen presentation pathway genes were not up-regulated in a coordinated fashion in response to rIFN- $\gamma$ 2. Therefore, the transcriptional response of MHC I-related genes was determined in AGD<sup>-</sup> NL gill cells stimulated with rIFN- $\gamma$ 2 (Fig. 7.9). Up-regulation in  $\gamma$ IP-10 ( $3.7 \pm 1.2$ ) confirms that the gill tissues could be stimulated with rIFN- $\gamma$ 2, however, no transcriptional response was observed from any MHC I-associated genes examined in this study.

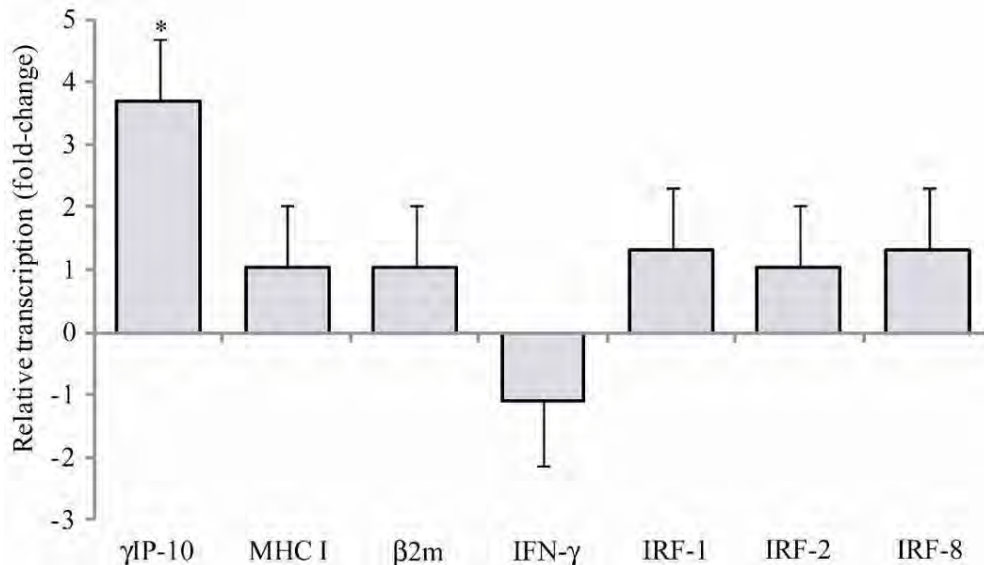


Figure 7.9. The expression of MHC class I (MHC I)-associated genes in Atlantic salmon, (*Salmo salar*) gill cells is not induced by short-term stimulation with recombinant Atlantic salmon interferon- $\gamma$  isoform 2 (rIFN- $\gamma$ 2). Transcriptional response of IFN- $\gamma$  and IFN- $\gamma$ -inducible genes associated with the MHC I antigen processing and presentation pathway in Atlantic salmon gill cells stimulated *ex vivo* for six hours with rIFN- $\gamma$ 2 (n = 7 fish). The relative transcription (fold-change) of genes in the gill tissues were determined by quantitative real-time PCR (qRT-PCR) analysis of mRNA. Data are presented as mean fold-change ( $\pm$ standard error). qRT-PCR threshold values were normalised against elongation factor 1 $\alpha$  and RNA polymerase II. Significant differences ( $p < 0.05$ ) in gene regulation are indicated with an asterisk (\*). Genes examined were IFN- $\gamma$ -inducible CXCL10-like protein ( $\gamma$ IP-10), MHC class I subunit  $\alpha$ 3 of the *Sasa*-UBA locus (MHC I),  $\beta$ 2-microglobulin ( $\beta$ 2m), IFN- $\gamma$ , interferon regulatory factor I (IRF-1), interferon regulatory factor-2 (IRF-2) and interferon regulatory factor-8 (IRF-8).



## 7.5 Discussion

The MHC I-restricted presentation of antigenic peptides on the cell surface is important for stimulating the host immune response to pathogens and the regulation of cell homeostasis. Despite the importance of the MHC I pathway, AGD-affected Atlantic salmon display a coordinated down-regulation in the MHC I antigen processing and presentation pathway within gill lesions (Young et al., 2008a). The simultaneous down-regulation of IRF-1, a transcriptional activator of the MHC I pathway suggested that the disruption in MHC I presentation was epigenetic in origin and therefore reversible. Whilst type I and type II IFNs can induce IRF-1 and other MHC I-associated genes, only the type II interferon IFN- $\gamma$  was down-regulated in AGD lesions and therefore proposed as a central regulator of the MHC I processing and presentation pathway in AGD lesions (Young et al., 2008a). The aim of this study was to ascertain whether IFN- $\gamma$  could restore “normal” MHC I expression in AGD lesions *ex vivo*. A second isoform of IFN- $\gamma$  (*Sasa*-IFN- $\gamma$ 2) was cloned from the gills of Atlantic salmon, sequenced and expressed and shown to be biologically active, up-regulating IRFs and MHC I in Atlantic salmon HKLs. Stimulation of dissociated AGD lesion cells with rIFN- $\gamma$  up-regulated  $\gamma$ IP-10 and IRF-1 yet failed to induce MHC I or  $\beta$ 2m expression. The co-expression of a known repressor of IRF-1-mediated transcription, IRF-8, in AGD lesions may have inhibited the IRF-1-mediated activation of MHC I transcription. Upon further investigation healthy gill was observed to respond to IFN- $\gamma$  stimulation although neither IRF-1 nor MHC I were induced. The apparent suppression of IFN- $\gamma$ -signalling in AGD lesions and the non-responsiveness of normal gills to IFN- $\gamma$  stimulation suggests that IFN- $\gamma$  alone is not a critical regulator of MHC I antigen presentation in the gills of Atlantic salmon.

In this study, dissociated gill cells were incubated with rIFN- $\gamma$  *ex vivo* for six hours in an effort to maintain their original cellular state (i.e. with down-regulated MHC I antigen presentation pathway) and ensure cell viability. Whilst the duration of IFN- $\gamma$  treatment was restricted, the expression of MHC I in the HKLs within a similar time frame indicated that a response within gills could be anticipated. Most importantly, a suppressed MHC I response in gills following IFN- $\gamma$  treatment is in agreement with previous studies showing that the expression of MHC I molecules remains stable in gills following pathogen-mediated IFN expression (Chang et al., 2005; Jørgensen et al., 2008; Jørgensen et al., 2007b; Landis et al., 2008) despite evidence for the IFN-mediated induction of the MHC I pathway in the head kidney (Jørgensen et al., 2007b; Ooi et al., 2008; Zou et al., 2005), liver (Jørgensen et al., 2008), spleen (Hansen and La Patra, 2002; Jørgensen et al., 2007b) and established head kidney cell lines (Jørgensen et al., 2006b; Martin et al., 2007a; Martin et al., 2007b; Martin et al., 2007c) of fish. . Therefore, this study adds to the growing evidence of a restricted response to IFN-signalling in the gills of fish.

The unresponsiveness of gills to IFN-signalling may result from its direct interaction with the aquatic environment. The gills of fish are constantly flushed with water that carries antigens derived from components of commensal flora and fauna or pathogenic organisms. It is likely that pattern recognition receptors on the surface of the gill are constantly engaged by an array of pathogen-associated molecular patterns (PAMPs). Despite this, healthy fish are capable of keeping the immune response in check in the face of persistent antigenic stimulus. In mammals it is recognised that counter-regulatory mechanisms maintain homeostasis in immune effector cells and, when activated by inflammation, minimise tissue damage caused by excessive or

inappropriate immune activation (Baker, 2006; Iweala and Nagler, 2006; Mellor and Munn, 2008; Romagnani, 2004). In these circumstances regions of immune privilege can develop, particularly when the ensuing cell-mediated innate or adaptive immune response may be more damaging than that derived from the pathogen. In mammals, this has been shown to occur in a number of tissues including the large mucosal surfaces of the gut that are constantly exposed to commensal and pathogen-derived antigenic signals (Iweala and Nagler, 2006; Mellor and Munn, 2008). The unresponsiveness of gill tissues to immunostimulation suggests that fish may maintain a similar form of acquired immune privilege within the gills as previously suggested (Young et al., 2008a). Whilst it is yet to be established that fish maintain sites of immune privilege that correspond to those observed in mammals the results of this study indicate that the gills may be a suitable model organ to explore the establishment and maintenance of potential sites of immune privilege in fish.

Functionally, the most important issue is whether *N. perurans* trophozoites benefit from reduced MHC I antigen processing and presentation within AGD lesions. The MHC I pathway is traditionally associated with the presentation of endogenously synthesised antigenic peptides to CD8<sup>+</sup> T cells (Kindt et al., 2007; Sprent and Schaefer, 1985). Therefore the importance of MHC I-mediated signalling in the activation of T cell effector function is dependent on whether parasites are intra- or extracellular. For instance, studies utilising MHC I-deficient mice confirmed that the MHC I antigen presentation pathway was critical for a protective immune response to intracellular parasites (Bertholet et al., 2006; Deepe, 1994; Denkers et al., 1993; Tarleton et al., 1996; Tarleton et al., 1992) whilst resistance to extracellular parasites was independent of the MHC I antigen presentation pathway (Hernandez et al., 1997;

Holland et al., 2005). *N. perurans* trophozoites are ectoparasitic (Adams and Nowak, 2001; Adams and Nowak, 2003; Dyková et al., 1995; Kent et al., 1988; Munday et al., 1990), hence it is unlikely that parasite-mediated modulation of the traditional MHC I antigen presentation pathway would help *N. perurans* evade immunosurveillance unless alternate antigen presentation pathways exist. In mammals cross-presentation is known to occur, whereby exocytosed antigens are presented in a MHC I-restricted manner and induce CD8<sup>+</sup> T cell effector function (Bertholet et al., 2006; Kurts et al., 1996; Rock and Shen, 2005; Song and Harding, 1996). In so doing, the host rapidly generates cytotoxic T lymphocytes (CTLs) that attack the pathogen and pathogen-infected cells. Therefore, if the cross-presentation of exogenous antigenic peptides occurs in fish, the capacity for *N. perurans* to modulate the MHC I antigen presentation pathway would reduce their sensitivity to CTL-mediated lysis (Bukowski and Welsh, 1985; Pereira et al., 1995) .

As well as its importance for regulating the immune response to pathogens, the MHC I antigen presentation pathway also plays a role in regulating cell homeostasis. MHC I molecules function as anti-tumour effectors via the MHC I-mediated presentation of an altered cell antigenic profile to CD8<sup>+</sup> T cells (Kindt et al., 2007). The down-regulation of MHC I antigen presentation is recognised as a mechanism that allows tumorigenic cells to escape from cell lysis mediated by CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) (Bubenik, 2004; Lanier and Phillips, 1996). Within AGD lesions there are profound cellular changes that result in the loss of gill architecture, primarily the proliferation of epithelial-like cells (Adams and Nowak, 2003; Dyková et al., 1995; Kent et al., 1988; Munday et al., 1990; Rodger and McArdle, 1996; Roubal et al., 1989). Proliferating cells within AGD lesions share characteristics with

tumourogenic cells (Morrison et al., 2006a) and, like tumourogenic cells, may also evade CTL-mediated inhibitory control by expressing fewer MHC I receptors on their cell surface. A characteristic of many tumours is that the down-regulation in the MHC I processing machinery is under epigenetic control and can be rapidly upregulated by IFN- $\gamma$  treatment (Drake et al., 2006; Kaplan et al., 1998; Shankaran et al., 2001). In this study, the unresponsiveness of gills to IFN- $\gamma$  stimulation prevented any comparison between the transcriptional response of AGD lesions and normal gills following IFN- $\gamma$  treatment. However, if MHC I down-regulation does contribute to the hyperplastic cellular response within AGD lesions then the results of this study suggest restoration of cell homeostasis appears to be independent of IFN- $\gamma$  in the gills of fish.

It is acknowledged that greater insight may have been achieved by differentiating between the transcriptional response of individual cell phenotypes that constitute AGD-lesion and normal gill tissue. In order to achieve this however, phenotypic markers that can differentiate between epithelial and mesenchymal cells in teleost fish require extensive validation since the extrapolation of expression patterns of cell phenotype markers in other vertebrates is not recommended (Groff et al., 1997). For instance, cytokeratins are almost exclusively restricted to epithelial cells in mammals and are an epithelial cell phenotype marker (Moll et al., 1982). However, in teleosts, cytokeratins are expressed by both epithelial and a variety of mesenchymal cells (Schaffeld and Marld, 2004). Therefore, this study was restricted to only addressing whether the MHC I antigen processing pathway could be restored within the AGD-lesion environment.

The IRF family of transcription factors are best known as key modulators of the transcriptional response to IFN signalling (Nguyen et al., 1997; Taniguchi et al., 2001). During this study a putative IRF-8 ortholog in teleosts was identified and it was shown that Atlantic salmon IRF-1, IRF-2 and IRF-8 are all inducible upon stimulation with IFN- $\gamma$  as observed in other vertebrates (Jungwirth et al., 1995; Nelson et al., 1993). IRF-8 is a known repressor of IRF-1-mediated MHC I transcription (Driggers et al., 1990; Nelson et al., 1993), therefore the coordinated up-regulation of IRF-8 with IRF-1 in AGD lesions may have suppressed the MHC I transcription. However, the unresponsiveness of gills to IFN-signalling limited the transcriptional response observed, and therefore any definitive conclusions regarding the mechanisms that attenuate IRF-1-mediated transcription in fish. The roles of the IRF family are now recognised as being remarkably diverse including the regulation of the innate immune response, development of various immune cells, cell growth, cell survival and oncogenesis (Tamura et al., 2008). Obviously further studies of IFN- $\gamma$  signal transduction and attenuation mechanisms mediated by IRF-1, IRF-2 and IRF-8 in Atlantic salmon would benefit from being performed in organs or cells that are more responsive to IFN- $\gamma$  signalling.

This study explored the role of IFN- $\gamma$  in the induction of MHC I antigen presentation in the gills of AGD-affected Atlantic salmon. Stimulating AGD lesions and normal gills from healthy Atlantic salmon with IFN- $\gamma$  did not induce expression of MHC class I/ $\beta$ 2m mRNAs indicating that alternative mechanisms may be employed by fish to regulate MHC I antigen presentation in the gills of fish. The importance of MHC I in the host defence against *N. perurans* remains uncertain unless cross-presentation is clearly shown to occur in fish. Further studies to assess whether teleost fish are able

to cross-present antigenic peptide could be conducted using gene knockout or gene silencing experiments. For instance, if cross-presentation occurs in fish, cells or tissues that are MHC II $\alpha$  and/or MHC II $\beta$  gene-deficient would retain the capacity to present exogenous antigenic peptides on the cell surface via MHC I molecules and activate a cytotoxic T cell response. Assuming MHC I antigen presentation is important in AGD pathogenesis, further studies using a soluble agonist (e.g. phorbol ester) to stimulate MHC I antigen presentation in AGD-affected and healthy Atlantic salmon *in vivo* and quantitation of the transcriptional response in gills and other organs would provide further insight into the mechanisms that regulate the MHC I signal transduction in AGD-affected fish.

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## **8 GENERAL DISCUSSION**

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## 8.1 The aetiology of amoebic gill disease (AGD)

Employing culture-dependent methodologies, numerous strains of *Neoparamoeba pemaquidensis* and *Neoparamoeba branchiphila* were isolated from amoebic gill disease (AGD)-affected fish (Caraguel et al., 2007a; Dyková et al., 2005b; Dyková et al., 2007; Fiala and Dyková, 2003). Since these strains were morphologically similar to the trophozoites intimately associated with AGD lesions it appeared that AGD may be a condition of mixed aetiology (Caraguel et al., 2007a; Dyková et al., 2005b; Kent et al., 1988; Wong et al., 2004). The initial aim of this thesis was to resolve AGD aetiology by developing molecular tools that taxonomically discriminated trophozoites intimately associated with AGD lesions *in situ*. Surprisingly, by means of a culture-independent approach, a new phylogenetic lineage of *Neoparamoeba* was identified based on 18S and partial 28S rRNA gene phylogenies (Chapter 2). This new phylogenetic lineage was determined to be the only amoeba associated with AGD lesions in tank and field-based populations of AGD-affected Atlantic salmon in Tasmania, Australia (Chapters 2 and 3). As a consequence, this *Neoparamoeba* phylogenetic lineage was described as a new species, *Neoparamoeba perurans*, on the basis of phylogenetic and virulence-related phenotypic divergence from other *Neoparamoeba*.

Identifying *N. perurans* as the aetiological agent of AGD in Tasmania represents a breakthrough in our understanding of the role of *Neoparamoeba* species as parasites. For instance, in light of the results presented throughout this thesis (Chapter 2, 3, 5, 6 and 7) it is likely that *N. perurans* was the predominant species of amoeba responsible for the experimental induction of AGD in previous studies (for example Adams and Nowak, 2004a; Bridle et al., 2003; Bridle et al., 2005; Bridle et al.,

2006a; Bridle et al., 2006b; Embar-Gopinath et al., 2005; Gross, 2007; Gross et al., 2004b; Gross et al., 2005; Leef et al., 2005a; Morrison et al., 2006a; Morrison et al., 2004; Morrison et al., 2005; Villavedra et al., 2005; Vincent et al., 2007; Vincent et al., 2009; Vincent et al., 2006; Zilberg et al., 2001). Moreover, it is also likely that previous studies comparing so-called “wild-type” and clonal cultured strains of *Neoparamoeba* spp. (Villavedra et al., 2007; Villavedra et al., 2005; Vincent et al., 2009) actually represent a comparison between virulent, non-cultured *N. perurans* and avirulent, cultured strains of *N. pemaquidensis* and *N. branchiphila*. This new perspective on the aetiology of AGD has stimulated a re-evaluation of some general characteristics of *Neoparamoeba* that will be discussed in more detail within this chapter.

### **8.1.1 Are *N. perurans* cosmopolitan protozoa that elicit AGD globally?**

AGD is known to affect cultured marine fish world-wide (Table 8.1). Following the discovery that *N. perurans* is the exclusive aetiological agent of AGD in Tasmania it seemed prudent to examine the aetiological role of *N. perurans*, *N. pemaquidensis* and/or *N. branchiphila* in other cases of AGD described overseas. Utilising species-specific molecular probes (Chapter 2) only *N. perurans* was identified as the aetiological agent of 36 confirmed cases of AGD examined from 5 host fish species cultured across 9 countries (Table 8.1). Recently, *N. perurans* was also identified in association with AGD- and proliferative gill disease-affected Atlantic salmon cultured in Norway (Nylund et al., 2008; Steinum et al., 2008). Taken together these data unequivocally support the role of *N. perurans* as a cosmopolitan aetiological agent of AGD.

Table 8.1 Fish species, geographical locations that AGD was diagnosed and confirmed aetiological agent.

Species	Location	First AGD diagnosis	Confirmed aetiological agent	Aetiology reference
Atlantic salmon ( <i>Salmo salar</i> )	Tasmania, Australia	(Munday et al., 1990)	<i>Neoparamoeba perurans</i>	Chapters 2, 3, 6 and 7
	Spain	(Munday et al., 2001) <sup>a</sup>	Unknown	
	Ireland	(Rodger and McArdle, 1996)	<i>N. perurans</i>	Chapter 3
	Scotland	Chapter 3	<i>N. perurans</i>	Chapter 3
	Chile	(Clark and Nowak, 1999; Howard and Carson, 1993b) <sup>a</sup>	<i>N. perurans</i>	Bustos, Young and Nowak (unpublished)
	France	(Clark and Nowak, 1999; Findlay et al., 1995) <sup>a</sup>	Unknown	
	Washington State, U.S.A	(Young et al., 2008c)	<i>N. perurans</i>	Chapter 3
Ayu ( <i>Plecoglossus altivelis</i> )	Norway	(Steinum et al., 2008)	<i>N. perurans</i>	(Steinum et al., 2008)
	Japan	Nakano, Ogawa, Crosbie and Nowak (unpublished)	<i>N. perurans</i>	Nakano, Ogawa, Crosbie and Nowak (unpublished)
Blue warehou ( <i>Seriolella brama</i> )	Tasmania, Australia	(Adams et al., 2008)	Unknown	
Brown trout ( <i>Salmo trutta</i> )	France	(Findlay et al., 1995) <sup>a</sup>	Unknown	
Chinook salmon ( <i>Oncorhynchus tshawytscha</i> )	New Zealand	(Howard and Carson, 1993b) <sup>a</sup>	<i>N. perurans</i>	Chapter 3
Coho salmon ( <i>Oncorhynchus kisutch</i> )	Washington State, U.S.A	(Kent et al., 1988)	Unknown	
European seabass ( <i>Dicentrarchus labrax</i> )	Mediterranean	(Dyková et al., 2000)	Unknown	
Mediterranean seabream ( <i>Sparus aurata</i> )	Mediterranean	(Athanasopoulou et al., 2002) <sup>a</sup>	Unknown	
Rainbow trout ( <i>Oncorhynchus mykiss</i> )	Tasmania, Australia	(Munday et al., 1990)	<i>N. perurans</i>	Chapter 3
	France	(Findlay et al., 1995) <sup>a</sup>	Unknown	
Sharpshnout seabream ( <i>Diplodus puntazzo</i> )	Mediterranean	(Dyková et al., 2000)	Unknown	
Turbot ( <i>Psetta maxima</i> )	Spain	(Dyková et al., 1995; Dyková et al., 1998)	<i>N. perurans</i>	Chapter 3
	South Africa	Mouton, Dyková, Young, Nowak (unpublished)	<i>N. perurans</i>	Mouton, Dyková, Young, Nowak (unpublished)

<sup>a</sup> Reported but AGD diagnosis not confirmed

Cosmopolitanism in microbial eukaryotes is frequently reported (see reviews Bass et al., 2007; Finlay, 2002). The capacity for an organism to sustain a global distribution pattern most likely arose from a combination of local adaptation, extremely high abundance and ubiquitous dispersal patterns which increase the probability of finding the same species in the same habitat type, wherever the habitat exists world-wide (Esteban et al., 2001; Finlay, 2002). Therefore, if *N. perurans* is truly cosmopolitan then strains found within different geographical regions should be genetically similar. Indeed, a comparison of the available 18S rRNA gene sequences from *N. perurans* strains identified in Tasmania, Norway and Chile provided no evidence of endemism (Table 8.2 and Chapter 4). The lack of geographical regional differentiation observed between *N. perurans* strains is concordant with the lack of regional or host differentiation between strains of *N. pemaquidensis*, *N. branchiphila* or *N. aestuarina* based on comparisons of sequences derived from either the 18S rRNA gene (Chapters 2 and 4 and Dyková et al., 2005b; Dyková et al., 2007) or the second internal transcribed spacer (ITS2, Chapter 4). Therefore, based on these genetic data, all *Neoparamoeba* species examined appear to have a cosmopolitan distribution within the marine environment, with strains from adjacent sites being no more similar to each other than they are to those from more distant sites.

To be considered a cosmopolitan organism, *Neoparamoeba* would also have to be widely distributed in the marine environments in order to sustain a global distribution pattern. This was proposed by Page (1973; 1983) when he referred to the ubiquity of *N. pemaquidensis* and *N. aestuarina* in marine environments. Nevertheless, the available data on the global distribution patterns of *Neoparamoeba* species do not agree with their classification as being ubiquitous within all marine environments. In

fact, the collection locations of *Neoparamoeba* infer that their distribution is temperature restricted, with a “tropical barrier” separating strains collected from the northern and southern hemispheres (Fig. 8.1). One explanation for this may be that the *Neoparamoeba* were translocated with their host. For instance, the presence of genetically-related fish pathogens in geographically isolated regions has been associated with the translocation of their host fish (Hansen et al., 2003; Vike et al., 2009). Three species of *Neoparamoeba* have been isolated from the gills of Atlantic salmon, a species which has been successfully introduced from the coastal waters of the northern Atlantic Ocean to Australia, Chile and western North America (Welcomme, 1988). Indeed, the regions where Atlantic salmon have been introduced correspond to the geographical distribution of *Neoparamoeba* species. However, the risks of translocation via an Atlantic salmon host are considered to be remote, at least in the Australian example, since the salmonid families used by the Australian salmon aquaculture industry were all translocated as eggs, well before introduction to seawater (Ward et al., 1994). Thus, in Australia it is presumed that Atlantic salmon were exposed to regionally-derived *Neoparamoeba* species. More likely, the “tropical barrier” is only simulated and is an artefact of the bias toward *Neoparamoeba* collection locations in regions where fish farming is affected by AGD (Chapters 2 and 3 and Crosbie et al., 2003; Dyková et al., 2000; Dyková et al., 2005b; Dyková et al., 2007; Fiala and Dyková, 2003). Therefore, the current distribution patterns of *Neoparamoeba* are more indicative of the global distribution patterns of maricultured salmonids than the true distribution of *Neoparamoeba* within the marine environments. In support of this notion is the isolation of strains of *N. aestuarina* within water samples from the Ross Sea in Antarctica (Moran et al., 2007) and a strain of *N. branchiphila* isolated from the blue crab (*Callinectes*

*sapidus*) in the Gulf of Mexico (Dyková et al., 2007). These data offer preliminary evidence that *Neoparamoeba* species are likely to be endemic within a wider geographical distribution than is currently reported.

The cosmopolitan nature of *N. perurans* is highly significant for the global mariculture industry, particularly within regions of significant finfish production in northern and southern hemispheres. Currently, cases of AGD are only reported from fish cultured in temperate regions (Table 8.1 and Figure 8.1). If *N. perurans* are indeed ubiquitous in the marine environments then it can be assumed that the regions in which AGD outbreaks are commonly reported (Figure 8.1) reflect a requirement for specific physico-chemical conditions or a critical mass of fish in order to induce disease (Adams and Nowak, 2003; Clark and Nowak, 1999; Douglas-Helders et al., 2004) rather than a variation in the abundance of *N. perurans* trophozoites in the water column. This view is supported by the absence of AGD in natural fish populations within the vicinity of sea-cages that contain AGD-affected Atlantic salmon (Douglas-Helders et al., 2002). In this instance, estimating the abundance of *N. perurans* in the vicinity of fish farms may not be as useful a predictor of AGD epizootics as the current industry practice of measuring the relative severity of gross gill changes in AGD-affected cultured marine fish (Adams et al., 2004).

The data presented in this thesis suggests *N. perurans* is the aetiological agent of AGD world-wide. The genetic similarity between strains of *N. perurans* in geographically isolated regions indicates that *N. perurans* may be ubiquitous in the marine environment. However, to confirm whether *Neoparamoeba* retain a truly

cosmopolitan distribution pattern, non-biased surveys of *Neoparamoeba* species within marine environments are required.

Table 8.2. Similarity amongst the aligned *Neoparamoeba perurans* partial 18S rRNA gene nucleotide sequences (465 bp) from different geographical locations derived from mean character differences (percent)

Country	Australia	Norway	Chile
Australia	97.0 - 99.5	95.7 - 99.5	97.4 - 98.7
Norway	-	97.2	96.3 - 98.7
Chile			-

Similarity matrix generated using the available *N. perurans* 18S rRNA gene sequences from Australia (Genbank accession numbers: EF216899-EF216905), Norway (EF474480 and EU326494) and Chile (EU424141)

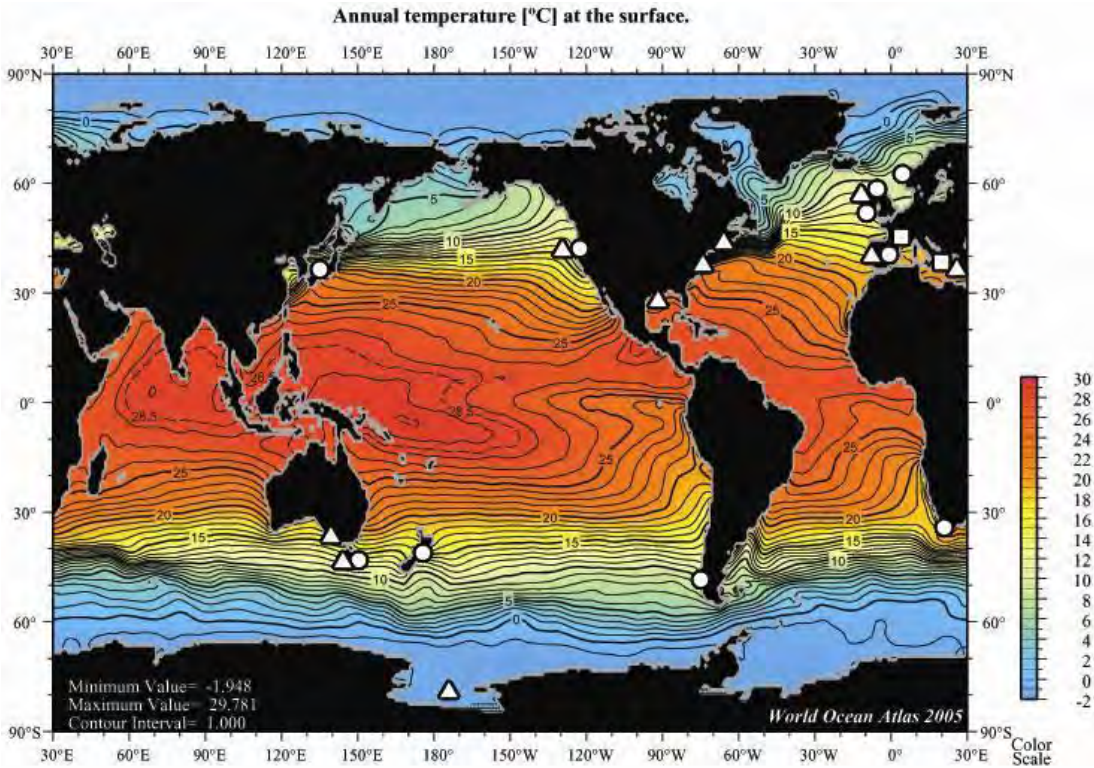


Figure 8.1. A “tropical barrier” separates northern and southern hemisphere strains of *Neoparamoeba*. Global distributions of collection locations for *Neoparamoeba* species in comparison to the 2005 mean annual sea surface temperature (<ftp://ftp.nodc.noaa.gov/pub/WOA05F/temperature/>). Confirmed cases of AGD induced by *Neoparamoeba perurans* are represented by a circle (●), unconfirmed cases of AGD with unknown aetiology are represented by a square (■) and the collection locations of *N. pemaquidensis*, *N. branchiphila* and *N. aestuarina* strains are represented by a triangle (Δ)

### 8.1.2 Are *Neoparamoeba* amphizoic?

*Neoparamoeba* are widely reported as amphizoic (for example Adams and Nowak, 2004b; Attard et al., 2006; Bermingham and Mulcahy, 2006; Caraguel et al., 2007a; Clark et al., 2003; Dyková et al., 2005b; Embar-Gopinath et al., 2005; Florent et al., 2007; Lee et al., 2006; Lovy et al., 2007; Powell et al., 2005; Wong et al., 2004).

This assumption is based on the premise that *Neoparamoeba* have the ability to exist as free-living organisms and infrequently colonise a host and live as parasites.

Indeed, successful isolation of *N. pemaquidensis* and *N. branchiphila* from sediment samples (Crosbie et al., 2003; Dyková et al., 2005b) and from a variety of host taxa (Caraguel et al., 2007a; Dyková et al., 2008; Dyková et al., 2000; Dyková et al., 2005b; Dyková et al., 2007; Fiala and Dyková, 2003; Kent et al., 1988; Morrison et al., 2005) constituted evidence that these species are amphizoic. However, a virulence-based phenotypic difference between *Neoparamoeba* species in relation to their capacity to induce AGD has now been demonstrated. For instance, despite a thorough examination of 36 samples of gill tissue sections from AGD-affected fish (Chapter 2, 3, 6 and 7 and unpublished data from our laboratory) neither *N. pemaquidensis* nor *N. branchiphila* were observed in close association with AGD-lesions nor un-affected tissue. Thus the possibility that these *Neoparamoeba* species remain as non-parasitic, free-living amoebae within the sampled gill tissues must be considered until conclusive evidence of parasitism is established. Conversely, *N. perurans* have only been identified as parasites in close association with AGD lesions (Chapter 2, 3, 5, 6 and 7 and unpublished data from our laboratory) with no evidence that they live independent of a host. Whilst it is premature to propose that *N. perurans* are obligate parasites, failed attempts to culture this species using methods optimised for other *Neoparamoeba* species (Dyková et al., 1995; Dyková et



al., 2000; Dyková et al., 2005b; Dyková et al., 2007; Fiala and Dyková, 2003; Kent et al., 1988; Morrison et al., 2005) suggests that host-derived factors may be required for their survival. Clarification of the life-history strategies employed by *N. perurans* would facilitate their shift to *in vitro* culture by anticipating their physico-chemical requirements. More broadly, clarification that *N. perurans* are amphizoic or obligate parasites would assist in the development of epidemiological studies by predicting the likely habitats where these amoebae may be found.

In discussing the potential for *Neoparamoeba* to be amphizoic it is also recognised that AGD is not the only disease associated with *Neoparamoeba*-like amoebae.

Amoebic diseases caused by *Paramoeba* or *Neoparamoeba* have been reported in invertebrates including the American lobster (*Homarus americanus*) (Mullen et al., 2004), blue crab (*C. sapidus*) (Johnson, 1977; Newman and Ward Jr., 1973; Sprague et al., 1969) and the sea urchin, *Strongylocentrotus droebachiensis* (Jones, 1985; Jones and Scheibling, 1985). Unfortunately no amoebae were cultured from these amoebic disease-affected animals and based on the limited morphological data that are available, most notably, the presence of parasomes, it is possible that the trophozoites associated with disease were *Paramoeba* or *Neoparamoeba* (Dyková et al., 2007; Mullen et al., 2005). In the case of amoebiasis in the American lobster, Mullen (2005) used 18S rRNA gene sequences that were PCR-amplified from fresh gill and antennae samples to infer that the aetiological agent was *N. pemaquidensis*. Therefore these results suggest that *N. pemaquidensis* may be amphizoic. However, as demonstrated throughout this thesis, inference of disease aetiology must be carefully assessed since morphologically similar *Neoparamoeba* species may be isolated from the gills of fish but only *N. perurans* elicits AGD.

Recently, *Neoparamoeba* were isolated from healthy invertebrate hosts including a strain of *N. branchiphila* from a blue crab [*C. sapidus*, (Dyková et al., 2007)] and sea urchin (*Heliocidaris erythrogramma*, (Dyková et al., 2007) ] and one strain of *N. aestuarina* from a sea urchin [*Paracentrotus lividus* (Dyková et al., 2008)].

Therefore, like fish, invertebrates without clinical signs of amoebic disease can also harbour *Neoparamoeba* and therefore a cautionary approach should be used to infer disease aetiology. Expanding the application of species-specific molecular tools developed in this thesis (Chapter 2 and 5) to the identification of trophozoites within invertebrate hosts would be highly informative in verifying the role of *N. pemaquidensis* as the aetiological agent in affected American lobster and examining the role of *Neoparamoeba* in epizootics affecting natural populations of invertebrates in general. Until the issue of amphizoism is resolved, it is recommended that researchers be cautious and refrain from assuming that *Neoparamoeba* are amphizoic.

## **8.2 Host response to AGD**

Atlantic salmon are highly susceptible to AGD which can lead to devastating stock losses if left untreated (Chapter 3 and Munday et al., 2001; Steinum et al., 2008).

Protective immunity against *N. perurans* appears to be inhibited, since to date, there is no definitive evidence that AGD-affected fish develop innate (Bridle et al., 2006a; Bridle et al., 2006b; Morrison et al., 2007) or acquired (Akhlaghi et al., 1996; Findlay and Munday, 1998; Gross, 2007; Gross et al., 2004b; Morrison et al., 2006a; Vincent et al., 2009; Vincent et al., 2006) immunity to the condition. This is supported by anecdotal evidence obtained from a continuous laboratory infection of

*N. perurans* maintained for 8 years in our laboratory, where none of the Atlantic salmon introduced into this system have ever developed resistance to AGD.

During this study, a molecular approach was used to identify mechanisms that underpin the susceptibility of Atlantic salmon to AGD. Based on the premise that the disruption of the host immune response is epigenetic in origin, the transcriptome of Atlantic salmon genes were profiled within AGD-affected tissues to identify the cellular mechanisms that contribute to AGD pathogenesis. Previous experiments that profiled the transcriptional response of AGD-affected Atlantic salmon (Bridle et al., 2006a; Morrison et al., 2006a; Morrison et al., 2007) were re-evaluated using an Atlantic salmon 16,000 gene microarray platform (von Schalburg et al., 2005). Employing a tissue-focussed approach, the transcriptional response was confirmed to be largely restricted to AGD lesions and permitted the identification of an interferon regulatory factor 1 (IRF-1)-mediated, interferon- $\gamma$  (IFN- $\gamma$ )-independent (Chapter 7) coordinated down-regulation in major histocompatibility complex class I (MHC I) and MHC class II (MHC II) antigen processing and presentation pathways within AGD lesions (Chapter 6). Suppressed mRNA expression of genes associated with the presentation of exogenous and endogenous antigens can lead to a reduced capacity of host cells to signal the attachment of a pathogen within affected tissues (Hewitt, 2003; Semnani et al., 2004; Tobian et al., 2003). Thus, it is proposed that molecular mechanisms important for the activation of an effector T cell-mediated response are disrupted in AGD lesions providing *N. perurans* with the capacity to evade the host immune response.

Transcriptome profiling of AGD-affected Atlantic salmon gill lesions also identified transcriptional modification of genes associated with changes in the tissue architecture and cellular homeostasis. For instance, epithelial cells appeared to be the predominant cell-type within AGD lesions (Chapter 6) and their hyperproliferative state may be mediated by the inhibition of the transcription factor p53 (Morrison et al., 2006a). Further discussion on the immunological characteristics of AGD lesions and how the development of these tissue microenvironments may contribute to the reduced capacity of Atlantic salmon to develop immunity to AGD are discussed in detail below.

### **8.2.1 Are AGD lesions sites of immune privilege?**

The immune system is equipped with potent effector mechanisms to eliminate pathogenic organisms and infected or tumourogenic cells (Kindt et al., 2007). The capacity to counter-regulate these effector mechanisms is necessary in order to maintain cell homeostasis and an appropriate immune response. This is of particular importance within regions requiring immune protection whilst avoiding inflammation and immunopathology that may threaten organ integrity or physiological functions (Iweala and Nagler, 2006; Streilein, 1993). In mammals, one method of contending with inappropriate immune activation is to create immunologically “privileged” sites whereby antigens elicit unexpectedly weak or no immune responses (Medawar, 1961). The concept of immune privilege (i.e. immunological tolerance) was originally restricted to particular mammalian organs (Medawar, 1961; Streilein et al., 1997) but is now applied to all localised tissue settings where varying levels of structural and functional integrity are preserved in the face of injury or danger (Forrester et al., 2008; Mellor and Munn, 2008).

It is assumed that teleost fish maintain a repertoire of mechanisms to counter-regulate an inappropriate immune response. For instance, Atlantic salmon appear to counter-regulate a transcriptional response to IFN- $\gamma$  signalling within their gills presumably to prevent an inappropriate immune response from affecting physiological function (Chapter 7). However, mechanisms that maintain an immunologically unresponsive state can be a double-edged sword, in that whilst avoiding inappropriate immune activation they can also provide opportunities for pathogens to evade host immunity (Belkaid et al., 2001; Lang et al., 2007; Peters and Sacks, 2006; Popov et al., 2006; Wilson and Crabtree, 2007). In AGD-affected Atlantic salmon gill lesions, immune mechanisms appear to be inactive and it is proposed that this may permit *N. perurans* to evade immunosurveillance (Chapters 6 and 7). Thus, Atlantic salmon AGD gill lesions share common characteristics of sites of immune privilege described in mammals such as a lack of classic acute inflammatory response in response to tissue insult (Mellor and Munn, 2008) and localised suppression of MHC receptor expression (Drake et al., 2006; Hong and Van Kaer, 1999).

In mammals, the entire suite of molecular mechanisms that underlie the active suppression of an immune response in sites of “privilege” are yet to be fully understood, however several mechanisms have now been described (Fig. 8.2). For instance, the suppression of MHC I expression on the cell surface can subvert the immune response by impairing T cell signalling pathways (Ito et al., 2004). The immune response can also be locally modified through the secretion of immunosuppressive cytokines such as tumour growth factor- $\beta$  (TGF- $\beta$ ) and

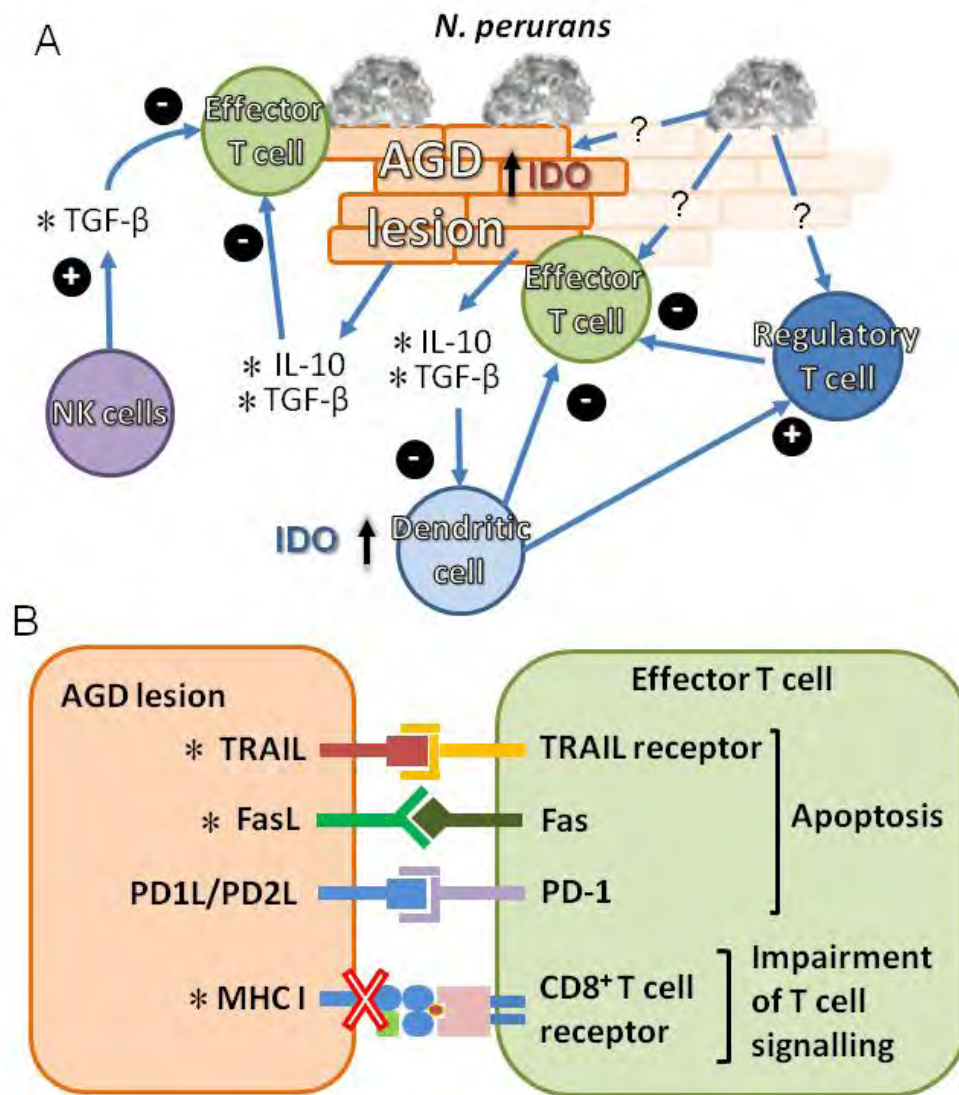


Figure 8.2. Common mechanisms found within sites of immune privilege that may disrupt effector T cell activity within amoebic gill disease (AGD) lesions. A. Common cell types and signal molecules [interleukin-10 (IL-10), transforming growth factor- $\beta$  (TGF- $\beta$ ) and indoleamine 2, 3-dioxygenase (IDO)] that regulate effector T cell function within sites of immune privilege. B. Receptors [tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), Fas ligand (FasL), programmed cell death 1 ligand (PD1L) and PD2L and major histocompatibility complex class I receptor (MHC I)] that may interact with effector T cells within AGD lesions and lead to the development of localised immune suppression. Atlantic salmon receptors and signal molecule transcripts that were assessed for changes in the levels of mRNA expression within AGD lesions are denoted by a star (\*).

interleukin-10 (IL-10) which suppress effector T cell activation (D'Orazio and Niederkorn, 1998). Alternatively, the expression of TNF-related apoptosis-inducing ligand (TRAIL), Fas ligand (FasL), programmed cell death 1 ligand (PD1L) and

PD2L on the surface of cells can work independently or synergistically to induce apoptosis of effector T cells (Mariani and Krammer, 1998; Okazaki and Honjo, 2006). Further, in certain circumstances over expression of enzymes with immunoregulatory effects, such as indoleamine 2, 3-dioxygenase (IDO) can also generate immune tolerance to foreign antigens (Katz et al., 2008).

In AGD-affected Atlantic salmon, focal sites of infection are associated with a down-regulation in the expression of MHC receptors. However, if it is presumed that AGD lesions are comparable to sites of immune privilege in mammals then additional mechanisms might also be used in AGD lesions to suppress the localised immune response. Therefore, preliminary examination of the transcriptional response of Atlantic salmon IL-10, TGF- $\beta$ , TRAIL and FasL within AGD lesions was performed (Fig. 8.3). Previously, the expression of TGF- $\beta$  mRNA was demonstrated to be stable within AGD-affected rainbow trout gill tissues compared to healthy gill tissues from AGD-naïve fish (Bridle et al., 2006b). However, as only the AGD lesion microenvironment is presumed to be immunosuppressed (Chapter 6 and 7) it was important to re-examine TGF- $\beta$  expression specifically within AGD-affected Atlantic salmon gill lesions. The same tissue samples used in Chapter 6 were analysed, allowing integration of these new results with data collected previously. The samples compared were gill lesions from AGD-affected fish (AGD<sup>+</sup> L) and non-lesion tissues from AGD-affected fish (AGD<sup>+</sup> NL) and AGD-naïve (AGD<sup>-</sup> NL) fish following 25 and 36 days post-exposure to *N. perurans* (Fig. 8.3). Neither of the immunosuppressive cytokine genes nor the apoptosis-inducing receptor genes were up-regulated in AGD lesions at a level greater than unaffected gill tissues (Fig. 8.3B and C). In fact, IL-10 appeared to be down-regulated in all AGD lesions at 25 days

post-exposure to *N. perurans* (Fig. 8.3B). Therefore, it is hypothesised that AGD lesions may be sites where tight immune-regulatory mechanisms operate but there appears to be no gross differences in the transcription of genes that are important in mammalian sites of immune privilege. To substantiate this hypothesis, further assessments using quantitative genomic (i.e. quantitative real-time PCR) and/or other methods (i.e. immunohistochemistry and ELISA) are required. It is also possible that other immunosuppressive mechanisms (see reviews Forrester et al., 2008; Hong and Van Kaer, 1999; Mellor and Munn, 2008) are important within the AGD lesion microenvironment. For instance, in mammals the recruitment of specific immunoregulatory cell populations is critical in maintaining immunosuppressive environments (Fig. 8.2). Several different subsets of CD4<sup>+</sup> T regulatory cells (T<sub>REG</sub> cells) are implicated in the induction of immunosuppression at the immune privilege site interface (see review Iweala and Nagler, 2006). Further, dendritic cells can induce effector T cell non-responsiveness in sites of immune privilege (Hawiger et al., 2001) and promote naive T cells to differentiate into T<sub>REG</sub> cells (Wakkach et al., 2003). A number of the molecules that are exclusively expressed by dendritic and T cell populations have been reported in fish (Hordvik et al., 1996; Hordvik et al., 2004; Laing et al., 2006; Lovy et al., 2009; Moore et al., 2005) although these immunoregulatory cells are yet to be definitively identified. Therefore, whilst the interaction between dendritic cells and T cell sub-populations may contribute to immune suppression within AGD lesions, confirmation will require further characterisation of the sub-populations of fish leucocytes.



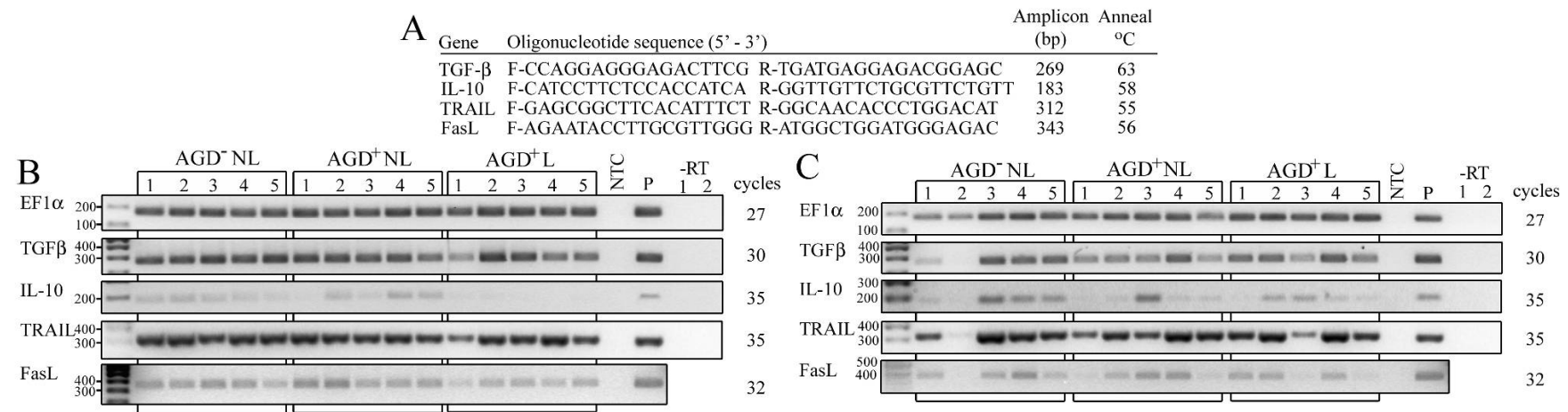


Figure 8.3. Gene markers for sites of immune privilege are not differentially regulated in amoebic gill disease-affected Atlantic salmon (*Salmo salar*) lesions. If the complete gene sequence was unavailable, a full length Atlantic salmon gene sequence was obtained by querying the ongoing Genomic Research on Atlantic salmon Project (GRASP) consortium clustering database (Rise et al., 2004b) using available teleost mRNA sequence data. Amino acid translations of open reading frames of each mRNA sequence were submitted to the PredictProtein server (Rost et al., 2004) which confirmed the presence of conserved motifs. Identity and similarity matrices were determined in BioEdit (Hall, 1999) using a BLOSUM62 amino acid substitution matrix. A. Forward (F) and reverse (R) oligonucleotide sequences designed to amplify an Atlantic salmon transforming growth factor-β [TGF-β, GenBank Accession number: ABH07955, (Ryynanen and Primmer, 2006)] sequence that shared 95.2% identity (ID) and 98.2% similarity (SIM) within the amino acid residues 111-382 of the 382 aa *Oncorhynchus mykiss* TGF-β (Hardie et al., 1998); Atlantic salmon interleukin-10 [IL-10, ABM46994, unpublished] sequence that shared 94.9% ID and 96.6% SIM from 40-157 aa residues of the 184 aa *O. mykiss* IL-10 (Inoue et al., 2005); an Atlantic salmon tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL, DQ218468 unpublished) sequence that shared 63.5% ID and 77.26% SIM to TRAIL from *Danio rerio* (Eimon et al., 2006); and an Atlantic salmon Fas ligand (FasL, ACI68894, unpublished) sequence that shared 42.5% ID and 58.42% SIM to FasL from *D. rerio* (Eimon et al., 2006). Atlantic salmon FasL and TRAIL mRNA sequences both retained their TNF family signature similar to ([LV]-x-[LIVM]-{V}-x-{L}-G-[LIVMF]-Y-[LIVMFY]2-x2-[QEKHL]-[LIVMG]-x-[LIVMFY]) [Prosit documentation PDOC00224, (Hulo et al., 2006)]. Elongation factor 1-α was used as an internal control (see Chapter 6 and 7). Reverse transcription PCR using cDNA samples described in Chapter 6 from AGD-affected gill lesions (AGD<sup>+</sup> L, n = 5 fish), non-lesion tissue from the same AGD-gill tissues (AGD<sup>+</sup> NL, n = 5 fish) and normal gill tissues from a AGD naive fish (AGD<sup>-</sup> NL, n = 5 fish) sampled at 25 (B) and 36 (C) days post-exposure to *Neoparamoeba perurans*. PCR reagents were identical to those used in Chapters 5. PCR cycle conditions using all pairs of oligonucleotides were 94°C for 30 sec; 94°C for 30 s, the specific annealing temperature described above (Figure 8.3 A) for 30 s and 72°C for 30 s for a gene-specific number of cycles; and 72°C for 10 min. The number of PCR amplification cycles for each reaction is provided on the right of each gel image (Fig. 8.3 B and C). Total RNA from two fish was reverse transcribed without reverse transcriptase as a control (-RT). Control PCR reactions included no DNA template control (NTC) and a positive control (P) consisting of cDNA template prepared for quantitative RT-PCR efficiency estimation (Chapter 6). PCR reactions were electrophoresed through 2% agarose/tris-borate EDTA buffer and visualized by staining with 1μg mL<sup>-1</sup> ethidium bromide.

Regardless of the putative immunosuppressive mechanisms employed within AGD lesions, the implications of this on host survival and response to infection are most important. By exploiting sites of immune privilege, pathogens can establish local environments in which host immunity is not permitted. Therefore even if a host response to pathogenic antigens is manifested systemically, a chronic infection may persist (Belkaid et al., 2001; Lang et al., 2007; Popov et al., 2006; Wilson and Crabtree, 2007). This could explain why antibodies to *N. perurans* that are generated by AGD-affected fish fail to provoke protective immunity (Gross et al., 2004a; Vincent et al., 2009; Vincent et al., 2006). These data are profoundly important for the development of treatments for AGD since vaccine or immunostimulant-based treatment strategies that activate pathogen-specific immunity would also have to overcome the local suppression that maintains immune privilege. Conversely, any interference with the mechanisms that develop as a consequence of an interaction between host and parasite may have undesirable effects, such as the development of an excessive immune reaction that leads to additional tissue pathology or disruption in physiological function. For instance, when granulomatous lesions formed by *Mycobacterium tuberculosis* were disrupted in lung tissues, this increased neutrophilic influx and caused significant necrosis (Saunders and Cooper, 2000).

### **8.2.2 How are the molecular mechanisms manipulated within the AGD lesion microenvironment?**

It is now unequivocal that the aberrant cellular response within AGD-affected Atlantic salmon gill lesions is epigenetic in origin. Disruptions in cellular pathways can often be linked to a central transcriptional activator. For instance, the transcription factor IRF-1 plays a central role in the transcriptional activation of

MHC I and MHC II genes in mammals (Hobart et al., 1997). This also appeared to be the case in AGD-affected Atlantic salmon with the coordinated down-regulation in MHC I and MHC II antigen processing and presentation-associated genes associated with the down-regulation of IRF-1 (Chapter 6 and 7). In addition, the hyperplastic state of epithelial cells within AGD lesions was possibly mediated by a down-regulation of the transcription factor, p53 tumour suppressor protein (Morrison et al., 2006a). However neither IRF-1 nor p53 act as transcriptional activators of one another (Harada et al., 1994; Tuck and Crawford, 1989) and nor do they explain the differential regulation of all genes affected within AGD lesions (Chapter 6 and 7 and Bridle et al., 2006a; Morrison et al., 2006a; Morrison et al., 2007). This discrepancy inspired further scrutiny of differentially regulated genes in AGD-affected Atlantic salmon gill tissues when compared to healthy AGD-naïve fish (Chapter 6, 7, 8 and Bridle et al., 2006a; Bridle et al., 2006b; Morrison et al., 2006a; Morrison et al., 2009 manuscript in preparation; Morrison et al., 2007; Wynne et al., 2008). Interestingly, a significant number of the down-regulated (<2 fold,  $p < 0.05$ ) or stably expressed genes in AGD lesions are transcriptionally activated by nuclear factor kappa-B (NF- $\kappa$ B) (Table 8.3).

NF- $\kappa$ B is a family of pleiotropic transcription factors present in almost all cell types and are involved in many biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis (Caamano and Hunter, 2002; Christman et al., 1998; Hanada and Yoshimura, 2002; Pahl, 1999). Indeed, inactivation of NF- $\kappa$ B would explain the disruption in the transcription of genes in response to AGD identified throughout this thesis and in previous studies (Table 8.3).

Whilst NF- $\kappa$ B appears to represent a central regulator of aberrant cellular signalling within AGD lesions some discrepancies were observed. Interleukin-1 $\beta$  (IL-1 $\beta$ ), cyclooxygenase-2 (COX-2) and interleukin-8 (IL-8) are all transcriptionally activated by NF- $\kappa$ B yet they were up-regulated within AGD lesions (Table 8.3). Rather than being a discrepancy, these genes emphasise the complexity of cellular signalling that results in gene transcription. Upon further inspection of the promoter elements of these three genes in mammals an additional transcription factor, CAAT/enhancer-binding protein $\beta$  (C/EBP $\beta$ ) can also activate these genes (Basak et al., 2005; Christman et al., 1998; Kunsch and Rosen, 1993) and C/EBP $\beta$  was up-regulated in AGD lesions (Chapter 6). Thus, IL-1 $\beta$ , IL-8 and COX-2 appear to be transcriptionally activated in AGD lesions via C/EBP $\beta$ , independent of NF- $\kappa$ B. Therefore, inactivation of NF- $\kappa$ B signalling in AGD lesions is still a plausible explanation for the disruption in the immune response and alteration of cellular architecture in AGD lesions.

Table 8.3. Target genes of nuclear factor- $\kappa$ B (NF- $\kappa$ B) measured in AGD-affected fish

Genes	Description	NF- $\kappa$ B reference <sup>a</sup>	Whole gill <sup>b</sup>	Reference	AGD lesion <sup>c</sup>	Reference
<i>Acute phase proteins</i>						
SAA-like protein	Serum amyloid A	(Li and Liao, 1991)	Stable	(Bridle et al., 2006a)	-	-
SAP-like protein	Serum amyloid P/pentraxin	(Basile et al., 1997)	Stable	(Bridle et al., 2006a)	On chip (stable) <sup>d</sup>	Chapter 6
<i>Cytokines/Chemokines</i>						
$\gamma$ IP-10	Interferon-inducible protein 10, $\alpha$ -chemokine	(Ohmori and Hamilton, 1993)	-	-	Stable	Chapter 7
IFN- $\gamma$	Type II interferon	(Sica et al., 1997)	-	-	Variable or down	Chapter 6, 7 and (Morrison et al., 2007)
IL-1 $\beta$	Interleukin-1 $\beta$	(Hiscott et al., 1993)	Up	(Bridle et al., 2006a)	Up	(Morrison et al., 2007)
IL-8	Interleukin-8	(Kunsch and Rosen, 1993)	Stable	(Bridle et al., 2006b)	Up	Morrison in prep
IL-10	Interleukin-10	(Eskdale et al., 1997)	-	-	Down	Chapter 8 (previous section)
TNF- $\alpha$	Tumor necrosis factor $\alpha$	(Shakhov et al., 1990)	Stable	(Bridle et al., 2006b)	Stable	(Morrison et al., 2007)
<i>Immunoreceptors</i>						
Ig heavy chain	Immunoglobulin	(Lin and Stavnezer, 1996)	-	-	Down	Chapter 6
MHC I	Classical MHC class I	(Johnson and Pober, 1994)	-	-	Down	Chapter 6 and 7
$\beta$ 2m	$\beta$ -2-microglobulin	(Israël et al., 1989)	Down	(Morrison et al., 2006a)	Down	Chapter 6, 7 and (Morrison et al., 2007)
<i>MHC-associated genes</i>						
PA28 $\beta$	Proteasome activator subunit 2 $\beta$	(Ossendorp et al., 2005)	-	-	Down	Chapter 6
Invariant chain (CD74)	MHC II-associated invariant chain protein	(Brown et al., 1994)	-	-	Down	Chapter 6
<i>Regulator of apoptosis</i>						
FasL	Inducer of apoptosis	(Matsui et al., 1998)	-	-	Stable	Chapter 8 (previous section)
<i>Transcription factors</i>						
IRF-1	Interferon regulatory factor-1	(Harada et al., 1994)	-	-	Down	Chapter 6 and 7
IRF-2	Interferon regulatory factor-2	(Harada et al., 1994)	-	-	Down	Chapter 7
p53	Tumour suppressor protein	(Wu and Lozano, 1994)	-	-	Down	(Morrison et al., 2006a)
<i>Stress response genes</i>						
COX-2	Cyclooxygenase, prostaglandin endoperoxide synthase	(Christman et al., 1998)	Stable	(Bridle et al., 2006b)	Up	Morrison in prep
Ferritin	Iron storage protein	(Kwak et al., 1995)	-	-	Stable	Chapter 6
iNOS	Inducible nitric oxide synthase	(Gookin et al., 2006)	Up	(Bridle et al., 2006b)	Stable	(Morrison et al., 2007)

<sup>a</sup> Reference providing evidence for a role of NF- $\kappa$ B in transcriptional regulation of the gene

<sup>b</sup> Transcriptional response in AGD lesions measured in random sample of the primary gill filaments

<sup>c</sup> Transcriptional response within AGD lesions specifically

<sup>d</sup> Gene was represented on the 16,000 gene microarray chip but not differentially regulated in AGD lesions

Elucidating a cause for the inactivation of NF- $\kappa$ B is complicated by the fact that several receptor and non-receptor mediated pathways are involved (Figure 8.4, Perkins, 2007). The most well studied receptor mediated pathways include interleukin-1 (IL-1 $\beta$ ), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and toll-like receptor 4 (TLR4) whereby engagement of these receptors by their respective cognate ligands triggers a cascade of events which releases NF- $\kappa$ B dimers from a cytoplasmic NF- $\kappa$ B–inhibitor of NF- $\kappa$ B (I $\kappa$ B) complex and their translocation to the nucleus where they regulate gene transcription (Figure 8.2, Karin and Ben-Neriah, 2000).

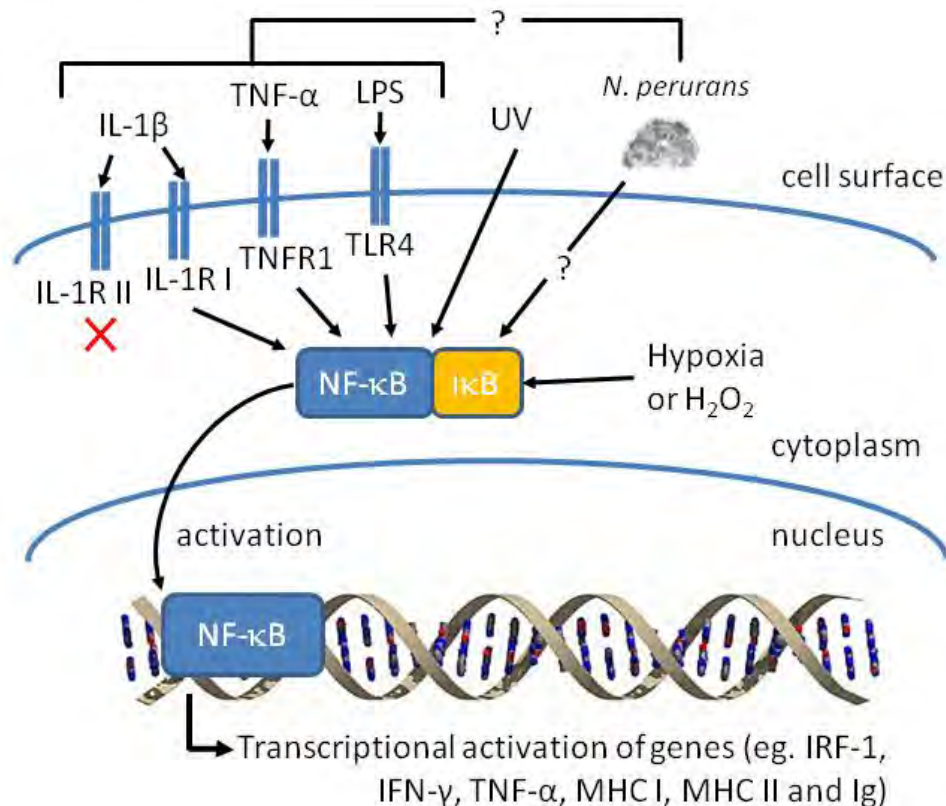


Figure 8.4. Common activation pathways of the nuclear factor- $\kappa$ B family of proteins. Receptor and non-receptor mediated signalling which release NF- $\kappa$ B dimers from a cytoplasmic NF- $\kappa$ B–inhibitor of NF- $\kappa$ B (I $\kappa$ B) complex and allows them to translocate to the nucleus where they regulate gene transcription. Examples of genes transcribed include those that were down-regulated in AGD-affected Atlantic salmon gill lesions. Receptor pathways that activate the NF- $\kappa$ B pathway include type I IL-1 receptor (IL-1RI) engagement by IL-1 $\beta$ , TNF receptor 1 (TNFR1) engagement by TNF- $\alpha$  and LPS binding to Toll-like receptor 4 (TLR4).

Surprisingly, interleukin-1 $\beta$  is highly expressed in AGD lesions (Bridle et al., 2006a; Morrison et al., 2007) and hence, should activate NF- $\kappa$ B-mediated gene transcription (Perkins, 2007). However, disruption in the IL-1 $\beta$  signalling pathway was identified by Morrison (2009 manuscript in preparation) to be associated with an increased expression of the type II IL-1 (IL-1R II) receptor relative to the type I IL-1 receptor (IL-1R I) in AGD lesions. In mice, binding of IL-1 to the IL-1R II does not activate NF- $\kappa$ B (Stylianou et al., 1992). Therefore, in the context of AGD lesions, failure of on-going expression of IL-1 $\beta$  to activate NF- $\kappa$ B may be associated with the subversion of IL-1 $\beta$ -signalling by preferential binding to the IL-1R II. Other pathways of NF- $\kappa$ B activation are yet to be investigated and it is possible that the alternate receptor-mediated and non-receptor-mediated pathways (Fig. 8.4) may contribute to the suppression of NF- $\kappa$ B mediated gene transcription observed in AGD lesions. It is also acknowledged that NF- $\kappa$ B may not be suppressed but rather cells within AGD lesions (i.e. hyperplastic epithelial cells) do not activate transcription of immunological genes (for example IRF-1, MHC I, TNF- $\alpha$ , iNOS) via NF- $\kappa$ B as would be anticipated in mammals (Pahl, 1999). Therefore, it is tentatively proposed that suppression of NF- $\kappa$ B-mediated transcription in AGD lesions is a means of rendering these tissues immunologically unresponsive. Certainly, a disruption in NF- $\kappa$ B-mediated transcription could explain the immune privilege-like state of AGD lesions. Whilst inactivation of NF- $\kappa$ B does not fit the current paradigm of immune privilege in mammals (Forrester et al., 2008; Iweala and Nagler, 2006; Mellor and Munn, 2008) the basic principal (immunological unresponsiveness) is upheld. Therefore, molecular mechanisms that maintain immunological unresponsiveness in AGD lesions may represent a novel strategy for maintaining a site

of immune privilege. However, additional information on the role of NF- $\kappa$ B-mediated transcription in healthy gill tissues is required in order to verify aberrant NF- $\kappa$ B activity within AGD lesions.

As mentioned, NF- $\kappa$ B is a central regulator in the recognition of an invading microorganism and is also associated with regulation of the subsequent immune response (Caamano and Hunter, 2002; Li and Verma, 2002). Therefore a large range of viral, bacterial and parasitic pathogens are able to target several parts of the NF- $\kappa$ B pathway, allowing them to interfere with the transcription of immune response genes thereby evading the host immune system (Hiscott et al., 2001; Kelly et al., 2004; Shapira et al., 2004; Tato and Hunter, 2002). Whilst most studies have focussed on the interaction of intracellular pathogens with the NF- $\kappa$ B system, there is evidence that extracellular parasites can also interfere with NF- $\kappa$ B activation. For instance the larval stage of the helminth parasite *Schistosoma mansoni* can interfere with NF- $\kappa$ B activation in endothelial cells to inhibit the recruitment of inflammatory cells to the lungs (Trottein et al., 1999). Based on these data, it is proposed that *N. perurans* may also subvert NF- $\kappa$ B mediated gene transcription to avoid the host immune system (Fig. 8.4). It remains uncertain how *N. perurans* may manipulate the immune response within AGD lesions although two explanations are proposed; A) IL-1 $\beta$ -mediated activation of NF- $\kappa$ B may be inhibited due to a shift in the ratio of the IL-1R I to IL-1R II on the cell surface and/or B) NF- $\kappa$ B activity is suppressed in comparison to baseline levels of NF- $\kappa$ B expression and activation in the gills of healthy fish, although further research is required in order to confirm this.



Since NF- $\kappa$ B is functionally activated via its translocation to the nucleus rather than via its transcription an alternative approach is required to pursue this research, focussing on measuring the interaction and location of proteins rather than the transcription of NF- $\kappa$ B associated genes. For instance, antibodies toward NF- $\kappa$ B-associated proteins could be used to determine whether NF- $\kappa$ B is in cytoplasmic (inactive) and/or nuclear (active) form and measure the relative expression of I $\kappa$ B protein within AGD-lesions. Alternatively, since rapid phosphorylation of I $\kappa$ B frees NF- $\kappa$ B to activate transcription (Karin and Ben-Neriah, 2000) it would also be possible to measure NF- $\kappa$ B activation by measuring the amount of I $\kappa$ B phosphorylation within AGD-lesions. Determining the mechanisms that *N. perurans* manipulate in order to inactivate NF- $\kappa$ B within AGD would greatly facilitate the development of preventative treatments for AGD. For instance, AGD treatments may require the reactivation of NF- $\kappa$ B-mediated transcription to stimulate immune surveillance within AGD lesions and allow the development of a protective immune response.

### **8.3 Conclusions**

Based on the data within this thesis, *N. perurans* is the only confirmed aetiological agent of AGD globally. Whilst being morphologically similar and phylogenetically related to other *Neoparamoeba* species it has diverged from other members of the group in being highly virulent and is a disease agent of global significance for marine aquaculture. These data were used within this thesis to confirm the aetiology of experimentally-

induced cases of AGD in Atlantic salmon and resulted in the development of diagnostic tools to pursue epidemiological and ecological studies into the life history strategies of *Neoparamoeba* and their distribution within the marine environments.

Data presented within this thesis contributes significantly to our understanding of the host response to AGD. It is proposed that the susceptibility of Atlantic salmon to AGD arises from the development of immune privilege-like sites within the AGD lesion microenvironment characterised by the suppression of MHC antigen presentation pathways. This immunological unresponsiveness to AGD may, in part, be a consequence of the need to tightly regulate the immune response within the gills of fish in order to avoid loss of physiological function due to inappropriate immune activation. Based on these hypotheses, the localised immunosuppression would explain the immunologically unresponsive state of AGD lesions and the lack of an acquired immunity to AGD. Whilst the data are preliminary, the development of AGD lesions may be induced via the inactivation of NF- $\kappa$ B signalling pathway permitting *N. perurans* to evade the host immune response. Finally it is proposed that an understanding of the mechanisms of localised immunosuppression will be particularly important for the development of new treatments for AGD since systemic immunostimulation may be ineffective without simultaneous disruption of the immune privilege-like microenvironment within AGD lesions.

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## APPENDICES

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### Appendix 1: Supplementary tables for microarray experiments (Chapter 6)

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As supplementary table 1 and 2 are large they were not embedded within this thesis.

Copies of supplementary tables 1 and 2 can be found on the disc attached to the back page of this thesis. Or, they can also be downloaded from:

<http://dx.doi.org/10.1016/j.molimm.2007.12.023>

**Appendix 2: Final copy of manuscripts published from this thesis can be found in pdf format on the disc attached.**

### **Appendix 3: Conference proceedings**

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Young ND, Nowak B, Morrison RN. Phylogeny of Atlantic salmon gill-derived amoebae. Aquafin CRC conference. Adelaide, South Australia, May 2007.

Young ND, Nowak B, Morrison RN. Transcriptome profiling the gills of AGD-affected Atlantic salmon: a tissue focussed approach. FRDC Aquatic Animal Health Subprogram Conference. Cairns, Queensland, July 2007.

Young ND, Nowak B, Morrison RN. Classification of gill-derived amoebae and diagnosis of amoebic gill disease (AGD). FRDC Aquatic Animal Health Subprogram Conference. Cairns, Queensland, July 2007.

Young ND, Nowak B, Morrison RN. Who killed the salmon? The phylogeny of gill derived amoebae. University of Tasmania Postgraduate Research Conference: Sharing Excellence in Research. Hobart, Tasmania, September 2007.

Young ND, Cooper G, Koop B, Nowak B, Morrison RN. Can *Neoparamoeba perurans* down-regulate antigen processing machinery in the gills of AGD-affected Atlantic salmon? Australian Society for Parasitology Conference. Glenelg, South Australia, July 2008.

Young ND, Nowak, B and Morrison RN. *Neoparamoeba perurans*, aetiological agent of amoebic gill disease. Australasian Aquaculture Conference. Brisbane, Queensland, August 2008.